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(57) Abstract

A method of effecting a gene therapy treatment in a host which comprises the steps of: (a) administering to a host concurrently (i) an adenoviral vector including at least one DNA sequence encoding a therapeutic agent and (ii) an immunosuppressive agent; (b) discontinuing the administration of said adenoviral vector and said immunosuppressive agent; and (c) repeating the administration of the adenoviral vector and the immunosuppressive agent at least once. The repeated course of treatment of administration of an adenoviral vector and an immunosuppressive agent provides for continued or increased expression of the at least one DNA sequence encoding the therapeutic agent.

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GENE THERAPY INVOLVING CONCURRENT AND REPEATED ADMINISTRATION OF ADENOVIRUSES AND IMMUNOSUPPRESSIVE AGENTS

This application is a continuation-in-part of Application Serial No. 08/478,482, filed June 7, 1995, which is a continuation-in-part of Application Serial No. 08/325,679, filed October 19, 1994, the disclosures of which are incorporated by reference.

This invention relates to gene therapy comprising the use of adenoviruses as the gene delivery vehicles. More particularly, this invention relates to gene therapy involving the concurrent and repeated administration of adenoviruses and immunosuppressive agents, whereby the efficiency of the gene therapy treatment is enhanced through suppression of an immune response against the adenoviruses.

BACKGROUND OF THE INVENTION

Adenovirus genomes are linear, double-stranded DNA molecules of approximately 36 kilobase pairs. Each extremity of the viral genome has a short sequence known as the inverted terminal repeat (or ITR), which is necessary for viral replication. The well-characterized molecular genetics of adenovirus render it an advantageous vector for gene transfer. Portions of the viral genome can be substituted

with DNA of foreign origin. In addition, recombinant adenoviruses are structurally stable.

Adenoviruses can be very efficient in gene transfer into cells in vivo, and, thus may be employed as delivery vehicles for introducing desired genes into eukaryotic cells, whereby the adenovirus delivers such genes to eukaryotic cells by binding cellular receptors. There are, however, several limitations to adenovirus gene transfer which are due in part to host responses directed at either the adenovirus vector particle, breakdown products of the vector particle, or the transduced cells. These host responses include non-specific responses and specific immune responses. The non-specific responses include inflammatory and non-inflammatory changes. An example of the latter is a change in host cell gene expression. Specific immune responses include various cellular responses and humoral antibody responses. Cellular responses include those mediated by T-helper lymphocytes, T-suppressor lymphocytes, cytotoxic T lymphocytes (CTL), and natural killer cells.

Despite the high efficiency of adenovirus vector mediated gene transfer, the transient nature of adenovirus vector mediated gene transfer has suggested that repeat administrations of adenovirus vectors may be necessary. Recent studies in cotton rats, however, have demonstrated that host immune responses directed towards adenoviral vectors correlate with decreased efficiency of gene transfer and expression after repeated administration. Yei *et al.*, *Gene Therapy*, 1:192-200 (1994).

Smith, *et al.*, Nature Genetics, Vol. 5, pgs. 397-402 (1993) discloses the administration to mice of an adenoviral vector including a human Factor IX gene. Such administration resulted in efficient liver transduction and plasma levels of

human Factor IX that would be therapeutic for hemophilia B patients. Human Factor IX levels, however, slowly declined to baseline by nine weeks after injection, and were not re-established by a second vector injection. Smith, *et al.*, also found that neutralizing antibodies to adenovirus block successful repeat administration of the adenovirus.

Kozarsky, *et al.*, J. Biol. Chem., Vol. 269, No. 18, pgs. 13695-13702 (May 6, 1994) discloses the infusion of an adenoviral vector including DNA encoding the LDL receptor to rabbits. Stable expression of the LDL receptor gene was found in the rabbits for 7 to 10 days, and diminished to undetectable levels within 3 weeks. The development of neutralizing antibodies to the adenovirus resulted in a second dose being completely ineffective.

Kass-Eisler, *et al.*, Gene Therapy, Vol. 1, pgs. 395-402 (1994) suggest that a T-cell response contributes to, but is not responsible solely for, the limited duration of expression in adults from adenovirus vectors. The authors further show that cyclosporin A is not effective in blocking the humoral response to the vector.

Fang, *et al.*, J. Cell. Biochem., Supplement 21A, C6-109, pg. 363 (1995) disclose the attempted re-injection of an adenovirus vector in dogs which were treated with cyclosporin A, an immunosuppressive agent. Such attempted re-injection was unsuccessful.

Yang, *et al.*, Proc. Nat. Acad. Sci., Vol. 91, pgs. 4407-4411 (May 1994) describe recombinant adenoviruses in which the E1a and E1b regions have been deleted. Such viruses also include a transgene. When such adenoviruses are administered to an animal host, cells harboring the recombinant viral genome express the transgene as desired; however, low level expression of viral genes also occurs. A virus-specific

cellular immune response is stimulated that leads to destruction of the genetically modified cells, thereby limiting the duration of expression of the transgene.

The aforementioned studies clearly document the need for a method of circumventing or blocking the host immune response to adenovirus vectors which prevents effective readministration of the vector, and limits the effectiveness of expression; however, they do not describe how to accomplish this.

It is therefore an object of the present invention to provide for sustained efficacy of gene transfer via repeated administration of adenoviral vectors, and for sustained expression of the transferred gene, through the suppression of an immune response against the adenoviral vectors.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention now will be described with respect to the drawings, wherein:

Figure 1 is a schematic of the construction of plasmid pHR.

Figure 2 is a schematic of the construction of an expression vehicle including an adenoviral ITR, an encapsidation signal. a Rous Sarcoma Virus promoter, an adenoviral tripartite leader sequence, and linking sequences;

Figure 3 is a schematic of the construction of plasmid pAvS6;

Figure 4 is a map of plasmid pAvS6;

Figure 5 is a map of plasmid pBQ4.7;

Figure 6 is a map of plasmid pAvS6 - CFTR;

Figure 7 is a schematic of adenoviral vectors Av1Luc1 and Av1Cf2;

Figure 8 is a map of plasmid pGEM-luc;

Figure 9 is a map of plasmid pAVS6-luc;

Figures 10A, 10B, and 10C depict the histologic appearance of the lung in response to Av1Cf2 administration three days after vector administration;

Figures 11A and 11B are graphs showing the effect of dexamethasone administration on lung lavage cells at 3 days and 42 days after the administration of Av1Cf2;

Figure 12 is a graph of anti-adenoviral antibody titers of lung lavage samples from rats infected with Av1Cf2 and which were treated or not treated with dexamethasone; and

Figure 13 is a graph of CTL responses in rats 42 days after infection with Av1Cf2;

Figure 14 is a graph of luciferase enzyme activity in rats infected with Av1Cf2 and which were treated or not treated with dexamethasone, followed by infection with Av1Luc1;

Figure 15 is a map of plasmid pAv1H9FR;

Figure 16 is a schematic of the adenoviral vector Av1H9F2;

Figure 17 is a graph of plasma human Factor IX levels in mice which were given Av1H9F2, and were or were not given one of the immunosuppressive agents deoxyspergualin, cyclophosphamide, or dexamethasone with or without the administration of the vector Av1LacZ4 five weeks earlier;

Figure 18 is a graph of plasma Factor IX levels (ng/ml) in mice which received from $1x10^3$ to $1x10^9$ pfu of Av1LacZ4, followed by administration of Av1H9FR five weeks later;

Figure 19 is a graph of neutralizing antibody titer in mice that were given Av1LacZ4, and received no immunosuppression, or were treated with deoxyspergualin, cyclophosphamide, or dexamethasone;

Figure 20 is a graph of plasma Factor VIII levels in mice which were given Av1LacZ4, and received no immunosuppression, or were given cyclophosphamide, followed by administration of Av1H9F2 with or without cyclophosphamide, followed by administration of Av1ALAPH81; and

Figure 21 is a graph of plasma Factor IX levels (ng/ml) in mice which received Av1LacZ4 and 0 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg, or 33 mg/kg deoxyspergualin, followed by administration of Av1H9F2.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with an aspect of the present invention, there is provided a method of effecting a gene therapy treatment in a host. The method comprises administering to a host (i) an adenoviral vector including at least one DNA sequence and (ii) an immunosuppressive agent. The course of administration of the adenoviral vector and immunosuppressive agent then is discontinued. Administration of the

immunosuppressive agent and the adenoviral vector then is repeated at least once. The adenoviral vector is administered in an amount effective to produce a therapeutic effect in the host. The immunosuppressive agent is administered in an amount effective to prevent or suppress a humoral and/or cellular immune response to the vector and/or cells containing the vector.

The term "DNA sequence" as used herein, refers generally to a polydeoxyribonucleotide molecule and more specifically to a linear series of deoxyribonucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of the adjacent pentoses.

Applicants have found that, when an immunosuppressive agent is administered with the adenoviral vector, and then administration of the vector is repeated, one achieves enhanced efficacy of the repeat *in vivo* adenoviral-mediated gene transfer through suppression of an immune response (such as a humoral antibody response) against the adenoviral vector and/or cells transduced with the vector, and thereby achieves increased expression of the transferred genes.

The adenoviral vector which is employed may, in one embodiment, be an adenoviral vector which includes essentially the complete adenoviral genome. Shenk *et al.*, *Curr. Top. Microbiol. Immunol.*, *111(3)*:1-39 (1984). Alternatively, the adenoviral vector may be a modified adenoviral vector in which at least a portion of the adenoviral genome has been deleted.

In another embodiment, the adenoviral vector comprises an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral encapsidation signal; at least one DNA sequence encoding a therapeutic agent; and a promoter controlling the at least

one DNA sequence encoding the therapeutic agent. The vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, but is not free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter.

In one embodiment, the vector also is free of at least a portion of at least one DNA sequence selected from the group consisting of the E2 and E4 DNA sequences.

In another embodiment, the vector is free of at least the majority of the adenoviral E1 and E3 DNA sequences, and is free of a portion of the other of the E2 and E4 DNA sequences.

In still another embodiment, the gene in the E2a region that encodes the 72 kilodalton binding protein is mutated to produce a temperature sensitive protein that is active at 32°C, the temperature at which the viral particles are produced. This temperature sensitive mutant is described in Ensinger *et al.*, *J. Virology, 10*:328-339 (1972); Van der Vliet, *et al.*, *J. Virology, 15*:348-354 (1975); and Friefeld *et al.*, *Virology, 124*:380-389 (1983); Englehardt, *et al.*, Proc.Nat. Acad. Sci., Vol. 91, pgs. 6196-6200 (June 1994); Yang, *et al.*, Nature Genetics, Vol. 7, pgs 362-369 (July 1994).

Such a vector, in a preferred embodiment, is constructed first by constructing, according to standard techniques, a shuttle plasmid which contains, beginning at the 5' end, the "critical left end elements," which include an adenoviral 5' ITR, an adenoviral encapsidation signal, and an E1a enhancer sequence; a promoter (which may be an adenoviral promoter or a foreign promoter); a multiple cloning site (which may be as hereinabove described); a poly A signal; and a DNA segment

which corresponds to a segment of the adenoviral genome. The vector also may contain a tripartite leader sequ nce. The DNA segment corresponding to the adenoviral genome serves as a substrate for homologous recombination with a modified or mutated adenovirus, and such sequence may encompass, for example, a segment of the adenovirus 5 genome no longer than from base 3329 to base 6246 of the genome. The plasmid may also include a selectable marker and an origin of replication. The origin of replication may be a bacterial origin of replication. Representative examples of such shuttle plasmids include pAvS6, shown in Figure 4. A desired DNA sequence encoding a clotting factor may then be inserted into the multiple cloning site to produce a plasmid vector.

This construct is then used to produce an adenoviral vector. Homologous recombination is effected with a modified or mutated adenovirus in which at least the majority of the E1 and E3 adenoviral DNA sequences have been deleted. Such homologous recombination may be effected through co-transfection of the plasmid vector and the modified adenovirus into a helper cell line, such as 293 cells, by $CaPO_4$ precipitation. Upon such homologous recombination, a recombinant adenoviral vector is formed that includes DNA sequences derived from the shuttle plasmid between the Not I site and the homologous recombination fragment, and DNA derived from the E1 and E3 deleted adenovirus between the homologous recombination fragment and the 3' ITR.

In one embodiment, the homologous recombination fragment overlaps with nucleotides 3329 to 6246 of the adenovirus 5 (ATCC VR-5) genome.

Through such homologous recombination, a vector is formed which includes an adenoviral 5' ITR, an adenoviral encapsidation signal; an E1a enhancer sequence; a promoter;

at least one DNA sequence encoding a therapeutic agent; a poly A signal; adenoviral DNA free of at least the majority of the E1 and E3 adenoviral DNA sequences; and an adenoviral 3' ITR. The vector also may include a tripartite leader sequence. This vector may then be transfected into a helper cell line, such as the 293 helper cell line (ATCC No. CRL1573), which will include the E1a and E1b DNA sequences, which are necessary for viral replication, and to generate infectious adenoviral particles. Transfection may take place by electroporation, calcium phosphate precipitation, microinjection, or through proteoliposomes.

The vectors hereinabove described may include a multiple cloning site to facilitate the insertion of the at least one DNA sequence encoding a therapeutic agent into the cloning vector. In general, the multiple cloning site includes "rare" restriction enzyme sites; i.e., sites which are found in eukaryotic genes at a frequency of from about one in every 10,000 to about one in every 100,000 base pairs. An appropriate vector in accordance with the present invention is thus formed by cutting the cloning vector by standard techniques at appropriate restriction sites in the multiple cloning site, and then ligating the DNA sequence encoding a therapeutic agent into the cloning vector.

The adenoviral vector, as stated hereinabove, includes at least one DNA sequence encoding at least one therapeutic agent. The term "therapeutic" is used in a generic sense and includes treating agents, prophylactic agents, and replacement agents.

DNA sequences encoding therapeutic agents which may be placed into the adenoviral vector include, but are not limited to, DNA sequences encoding tumor necrosis factor (TNF) genes, such as TNF-α; genes encoding interferons such

as Interferon-α, Interferon-ß, and Interferon-γ ; genes encoding interleukins such as IL-1, IL-1ß, and Interleukins 2 through 14; genes encoding GM-CSF; genes encoding adenosine deaminase, or ADA; genes encoding antioxidants such as Mn-SOD, catalase, CuZnSOD, extracellular superoxide dismutase, and glutathione reductase; genes which encode cellular growth factors, such as lymphokines, which are growth factors for lymphocytes; genes encoding growth factors such as epithelial growth factor (EGF) and keratinocyte growth factor (KGF); genes encoding soluble CD4; Factor VIII; Factor IX; von Willebrand's factor; T-cell receptors; the LDL receptor, ApoE, ApoC, ApoAI and other genes involved in cholesterol transport and metabolism; the alpha-1 antitrypsin (α1AT) gene, the ornithine transcarbamylase (OTC) gene, the CFTR gene, lung surfactant protein genes, the B-glucuronidase gene, the insulin gene, negative selective markers or "suicide" genes, such as viral thymidine kinase genes, such as the Herpes Simplex Virus thymidine kinase gene, the cytomegalovirus virus thymidine kinase gene, and the varicella-zoster virus thymidine kinase gene; Fc receptors for antigen-binding domains of antibodies, and antisense sequences which inhibit viral replication, such as antisense sequences which inhibit replication of hepatitis B or hepatitis non-A non-B virus; tissue plasminogen activator (tPA); urinary plasminogen activator (urokinase); hirudin; nitric oxide synthase; vasoactive peptides; and angiogenic peptides.

The DNA sequence encoding a therapeutic agent is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the Rous Sarcoma Virus (RSV) promoter; inducible promoters, such as the MMTV promoter, the metallothionein

promoter; heat shock promoters; the albumin promoter; and the ApoAI promoter. Alternatively, the DNA sequence encoding a therapeutic agent may be under the control of its native promoter. It is to be understood, however, that the scope of the present invention is not to be limited to specific foreign genes or promoters.

Immunosuppressive agents which may be employed include those which prevent: (i) a humoral (antibody) response against the adenoviral vector; (ii) a cellular immune response, such as, for example, a T-cell response to cells containing the adenoviral vector; or (iii) a non-specific inflammatory responses against the vector and against cells containing the vector. By preventing a humoral and/or T-cell and/or non-specific inflammatory response against the vector, and/or cells containing the vector administration of the immunosuppressive agent permits effective re-administration of the vector in order to produce a therapeutic effect in the host. Preferably, where repeat administration of the adenoviral vector is desired, the immunosuppressive agent is an immunosuppressive agent which prevents a humoral antibody response against the adenoviral vector. Preferably, where longer duration or higher levels of expression is desired, the immunosuppressive agent is one that prevents or suppresses a cellular or non-specific inflammatory response.

Host immune responses to *in vivo* adenovirus vector administration vary in relation to (i) the dose of vector; (ii) the route of administration; (iii) the level of replication (if any occurs); (iv) the nature of the transgene contained in the recombinant vector; (v) the genetic and physiological characteristics of the host; and (vi) the existence and level of pre-existing immune responses to previously administered adenovirus vectors.

In general, host responses are dependent on the dose of vector administered. Importantly, the magnitude of specific

host responses is dependent on the route of vector administration. For example, intravenous administration will yield a higher host antibody response than that of an equivalent amount of vector given via the respiratory route.

The diverse host responses to adenovirus vectors occur due to separate inflammatory and immune effector mechanisms, although most, if not all, of these distinct molecular mechanisms are connected and significantly interdependent. Thus, for example, humoral antibody formation is very dependent on certain T-helper lymphocyte support. Also, some cell-mediated cellular toxicity is dependent on antibody formation, e.g., opsonized macrophage cell killing.

The two principal host responses affecting the duration of transgene expression are the inflammatory response and the cellular immune response.

Inflammation is one of the first host responses that occurs following vector administration. Cytokine release is very likely involved in the subsequent influx of inflammatory cells. Such cytokines likely include IL-1, IL-6, IL-8, and TNF.

The amount of inflammation seen following in vivo adenoviral vector administration increases with incresing doses of vector given. Higher doses lead to a more rapid decline in transgene expression than do smaller doses.

CTL responses directed towards the transduced cells are believed to be important in reducing the duration of transgene expression. It is believed that the CTL are directed against low level adenovirus gene expression by the cells, which induces the CTL.

The principal host response affecting the ability to administer adenovirus vectors repeatedly to a host is the humoral antibody response. It develops to adenovirus

administered by a variety of routes, including oral, intravenous, intraperitoneal, and intrapulmonary. In general, the level of antibody response achieved is very dependent on the dose of vector administered. The antibody response is also dependent on the route of vector administration. Intravenous vector administration results in higher antibody levels than pulmonary administration for a given dose of vector. In contrast, wild type adenovirus elicits high antibody levels irrespective of the amount of virus given due to virus replication in vivo. The ability to repeat successfully adenovirus vector administration is inversely correlated with the level of circulating anti-adenovirus vector antibody present.

Pharmacologic modulation of host immune responses to adenovirus vectors involves the use of anti-inflammatory agents, celllular immune modifiers, and humoral antibody immune modifiers.

Anti-inflammatory agents include steroids, cyclophosphamide, and azothiophrine.

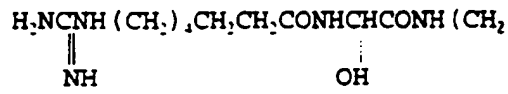
Steroids have potent anti-inflammatory properties. Applicants have shown that steroids, such as dexamethasone, given parenterally prolong the duration of transgene expression following in vivo administration of vector via the lung route. Steroids also block the function of lymphocytes. Thus, dexamethasone reduces the CTL responses observed after pulmonary vector administration. Dexamethasone also blocks, at least in part, the host antibody response to adenovirus vector administration.

Antibodies directed at cellular components of the immune system reduce cellular immune response. For example, anti T-cell receptor antibody (such as, for example anti-CD4 and anti-CD3 antibodies) administration prolongs transgene expression. CTLA4 immunoglobulin is another example. Anti-

CD4 antibody is directed against the T-helper lymphocytes and reduces their function. Other agents directed primarily at the cellular immune response include cyclosporins such as cyclosporin A; rapamycin binding protein ligands such as FK506; and steroids such as dexamethasone.

Agents which affect humoral antibody responses are generally directed at antibody producing B lymphocytes (B-cells) or at the T-cells which are responsible for inducing B-cell antibody production to high levels.

Examples of immunosuppressive agents which prevent a humoral antibody response against the adenoviral vector include, but are not limited to, deoxyspergualin, or DSG, which has the following structure:



The terms "deoxyspergualin and "DSG" as used herein, mean deoxyspergualin or DSG and derivatives or analogues thereof, such as salts of deoxyspergualin, including but not limited to, trihydrochlorides thereof, and any other analogues which have immunosuppressive activity. Such compounds are described further in U.S. Patent Nos. 4,525,299; 4,847,299; 5,162,581; and 5,196,453.

In one embodiment, the immunosuppressive agent which prevents a humoral antibody response is a steroid. Steroids which may be employed include, but are not limited to, dexamethasone, and any adrenocortical hormones, such as,for example corticosteroids; hydrocortisone; prednisolone; and methylprednisolone.

In another embodiment, the immunosuppressive agent which prevents a humoral antibody response is a cyclosporin, such as, for example, cyclosporin A. Other immunosuppressive agents which prevent a humoral antibody response and which may be employed include, but are not limited to,

azathioprine; cyclophosphamide; brequinar; leflunomide; mycophenolate mofetil; anti-CD40 antibody; anti-CD40 ligand antibody; cyclophosphamine; rapamycin; anti-CD4 antibody; CTLA-4 immunoglobulin; Interleukin -12; Interferon -γ; rapamycin binding protein (FEBP) ligands, such as, for example, FK506, as described in Bierer, *et al.*, Proc. Nat. Acad. Sci., Vol. 87, pgs. 9231-9235 (1990); Dumont, *et al.*, J. Immunol., Vol. 144, pgs. 1418-1424 (1990); and Bierer, *et al.*, Science, Vol. 250, pgs. 556-559 (1990); anti-lymphocyte function antigen-1 (LFA-1) antibody; and anti-T-cell receptor antibody.

Applicants have found that, when compounds which prevent, suppress, or eliminate humoral immune responses to foreign antigens (such as, for example, deoxyspergualin, cyclophosphamide, brequinar, leflunomide, mycophenolate, mofetil, anti-CD40 antibody, or anti-CD40 ligand antibody) are administered at a short time prior to, and/or during, and/or for a short time after adenoviral vector administration, to a host, such compounds prevent the production of anti-adenoviral neutralizing antibodies in the host. The prevention of the production of such neutralizing antibodies enables the efficient re-administration of the adenoviral vector to the host.

It is to be understood that, within the scope of the present invention, that an immunosuppressive agent may prevent more than one of the immune responses hereinabove described. It also is to be understood, however, that the scope of the present invention is not intended to be limited to any specific immunosuppressive agents.

It is also contemplated that within the scope of the present invention, a combination of immunosuppressive agents may be employed.

The adenoviral vector and immunosuppressive agent, in general, are administered concurrently in an amount effective to produce a th rapeutic effect in the host while preventing an immune response against the vector or against cells transduced with the vector. The term "concurrently," as used herein, means that the administration of the adenoviral vector and administration of the immunosuppressive agent are begun at approximately the same time, i.e., within a brief time frame of each other, and the administration of the adenoviral vector and the administration of the immunosuppressive agent are parts of a unitary course of treatment. Thus, for example, the immunosuppressive agent is administered at approximately the same time the adenoviral vector is administered, i.e., the administration of the immunosuppressive agent is begun at a short time (for example, about 24 hours) before, or during, or at a short time (e.g., 24 hours) after the administration of the adenoviral vector. In general, the immunosuppressive agent is administered according to standard dosage schedules established for that agent, and for a period of time which in general does not exceed 14 days, and preferably does not exceed 11 days, and more preferably does not exceed 8 days. Thus, long-term administration of the immunosuppressive agent is not required for enabling repeated administration of the adenovirus.

At the conclusion of the course of administration of the immunosuppressive agent, the course of administration of the adenoviral vector and immunosuppressive agent is discontinued for a period of time. The period of time between courses of administration of the adenoviral vector and the immunosuppressive agent, and the number of courses of administration of the adenoviral vector and immunosuppressive agent is dependent upon a variety of factors, including the age, weight, and sex of the patient, the disease or disorder

being treated, and the severity of the disease or disorder being treated.

It is to be understood that the above course of administration of the immunosuppressive agent is repeated with each administration of the adenoviral vector.

In one embodiment, the adenoviral vector may be administered, at each administration, in an amount of from 1 plaque forming unit to about 10^{14} plaque forming units, preferably from about 10^{6} plaque forming units to about 10^{13} plaque forming units, more preferably from about 10^{8} to about 10^{10} plaque forming units per kg. The host may be a human or non-human animal host.

The adenoviral vector may be administered systemically or topically. Examples of systemic administration include, but are not limited to, intravenous administration (such as for example, portal vein injection or peripheral vein injection), intramuscular administration, intraperitoneal administration, intranasal administration, or encapsulated oral administration.

The immunosuppressive agent is administered in an amount effective to produce a desired immunosuppressive effect in the host. The immunosuppressive agent may be administered, at each administration, in an amount of from about 1 mg/kg to about 15 mg/kg, when dexamethasone is employed, or at the dose equivalents for other steroids. When deoxyspergualin is employed, the deoxyspergualin may be administered in an amount of from about 1 mg/kg to about 33 mg/kg, preferably from about 3 mg/kg to about 7 mg/kg. When cyclophosphamide is employed, the cyclophophamide may be administered in an amount of from about 5 mg/kg to about 300 mg/kg, preferably from about 50 mg/kg to about 100 mg/kg.

The adenoviral vector particles and the immunosuppressive agent each may be administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid carrier such as, for example, a saline solution. The adenoviral vector particles also may be administered in combination with a solid carrier, such as, for example, microcarrier beads, or a sustained drug delivery material, such as, for example, a polyol.

Cells which may be transduced by the adenoviral particles include, but are not limited to, lung, airway, or alveolar epithelial cells; primary cells, such as primary nucleated blood cells, such as leukocytes, granulocytes, monocytes, macrophages, lymphocytes (including T-lymphocytes and B-lymphocytes), totipotent stem cells, and tumor infiltrating lymphocytes (TIL cells); bone marrow cells; endothelial cells; activated endothelial cells; epithelial cells; keratinocytes; stem cells; hepatocytes, including hepatocyte precursor cells; fibroblasts; mesenchymal cells; mesothelial cells; parenchymal cells; vascular smooth muscle cells; brain cells and other neural cells; gut enterocytes; gut stem cells; and myoblasts.

In one embodiment, the adenoviral particles may be targeted to blood cells, whereby such adenoviral vector particles infect the blood cells with a gene which directly or indirectly enhances the therapeutic effects of the blood cells. The gene carried by the blood cells can be any gene which allows the blood cells to exert a therapeutic effect that it would not ordinarily have, such as a gene encoding a clotting factor useful in the treatment of hemophilia. The gene can encode one or more products having therapeutic effects. Examples of suitable genes include those that encode the CFTR gene; cytokines such as TNF, interleukins

(interleukins 1-14), interferons (α, ß, γ-interferons), T-cell receptor proteins and Fc receptors for antigen-binding domains of antibodies, such as immunoglobulins. Other examples of suitable genes include genes encoding soluble CD4 which is used in the treatment of AIDS and genes encoding α-antitrypsin, which is useful in the treatment of emphysema caused by α-antitrypsin deficiency.

The transduced cells are useful in the treatment of a variety of diseases including but not limited to, cystic fibrosis, adenosine deaminase deficiency, sickle cell anemia, thalassemia, hemophilia, diabetes, α-antitrypsin deficiency, brain disorders such as Alzheimer's disease, phenylketonuria and other illnesses such as growth disorders and heart diseases, for example, those caused by alterations in the way cholesterol is metabolized and defects of the immune system.

In another embodiment, the adenoviral vector particles may transduce liver cells, and such adenoviral vector particles may include gene(s) encoding polypeptides or proteins which are useful in prevention and therapy of an acquired or an inherited defect in hepatocyte (liver) function. For example, they can be used to correct an inherited deficiency of the low density lipoprotein (LDL) receptor, and/or to correct an inherited deficiency of ornithine transcarbamylase (OTC), which results in congenital hyperammonemia.

In another embodiment, the adenoviral particles may transduce liver cells, whereby the adenoviral particles include a gene encoding a therapeutic agent employed to treat acquired infectious diseases, such as diseases resulting from viral infection. For example, the infectious adenoviral particles may be employed to treat viral hepatitis,

particularly hepatitis B or non-A non-B hepatitis. For example, an infectious adenoviral particle containing a gene encoding an antisense gene could be employed to infect liver cells to inhibit viral r plication. In this case, the infectious adenoviral particle, which includes a vector including a structural hepatitis gene in the reverse or opposite orientation, would be introduced into liver cells, resulting in production in the infected liver cells of an anti-sense gene capable of inactivating the hepatitis virus or its RNA transcripts. Alternatively, the liver cells may be infected with an infectious adenoviral particle which includes a gene which encodes a protein, such as, for example, α-interferon, which may confer resistance to the hepatitis virus.

The vector particles also may be employed in treating Hodgkin's lymphoma. An infectious adenoviral vector particle may be targeted to neoplastic cells of Hodgkin's lymphoma. The adenoviral vector particle also includes a negative selective marker or "suicide gene, such as the Herpes Simplex thymidine kinase gene. The adenovirus may be administered in vivo to a patient, whereby the virus infects neoplastic cells of Hodgkin's lymphoma. After the adenovirus is administered to the patient, the patient is given an interaction agent such as gancyclovir or acyclovir, whereby the neoplastic Hodgkin's lymphoma cells infected with the adenovirus are killed.

In addition, a vector may be constructed which includes the CFTR gene. The vector then may be administered to the respiratory epithelium in an effective therapeutic amount for the correction of the pulmonary deficit in patients with cystic fibrosis. In another example, vectors containing functional proteins may be delivered to the respiratory epithelium in order to correct deficiencies in such proteins.

Such functional proteins include antioxidants, α-1-antitrypsin, CFTR, lung surfactant proteins, cytokines, and growth factors such as EGF and KGF, and may also include adenosine deaminase for treatment of severe combined immune deficiency, von Willebrand's factor for treatment of Christmas disease, and β-glucuronidase for treatment of Gaucher's disease. Also, vectors including genes encoding anti-cancer agents or anti-inflammatory agents may be administered to lung cells of a patient for the treatment of lung cancer or inflammatory lung disease.

EXAMPLES

The invention now will be described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

Example 1

Construction of Av1Cf2 and Av1Luc1

A. Construction of pAvS6

The adenoviral construction shuttle plasmid pAvS6 was constructed in several steps using standard cloning techniques including polymerase chain reaction based cloning techniques. First, the 2913 bp BglII, HindIII fragment was removed from Ad-dl327 and inserted as a blunt fragment into the XhoI site of pBluescript II KS- (Stratagene, La Jolla, CA) (Figure 1).

Ad-dl327 is identical to adenovirus 5 except that an XbaI fragment including bases 28591 to 30474 (or map units 78.5 to 84.7) of the Adenovirus 5 genome, and which is located in the E3 region, has been deleted. The E3 deletion in Ad-dl327 is similar to the E3 deletion in Ad-dl324, which is described in Thimmappaya *et al.*, *Cell*, *31*:543 (1983). The complete Adenovirus 5 genome is registered as Genbank accession #M73260, incorporated herein by reference, and the

virus is available from the American Type Culture Collection, Rockville, Maryland, U.S.A. under accession number VR-5.

Ad-dl327 was constructed by routine methods from Adenovirus 5 (Ad5). The method is outlined briefly as follows and previously described by Jones and Shenk, *Cell 13*:181-188, (1978). Ad5 DNA is isolated by proteolytic digestion of the virion and partially cleaved with Xba I restriction endonuclease. The Xba I fragments are then reassembled by ligation as a mixture of fragments. This results in some ligated genomes with a sequence similar to Ad5, except excluding sequences 28591 bp to 30474 bp. This DNA is then transfected into suitable cells (*e.g.* KB cells, HeLa cells, 293 cells) and overlaid with soft agar to allow plaque formation. Individual plaques are then isolated, amplified, and screened for the absence of the 1878 bp E3 region Xba I fragment.

The orientation of this fragment was such that the BglII site was nearest the T7 RNA polymerase site of pBluescript II KS and the HindIII site was nearest the T3 RNA polymerase site of pBluescript II KS. This plasmid was designated PHR. (Figure 1).

Second, the ITR, encapsidation signal, Rous Sarcoma Virus promoter, the adenoviral tripartite leader (TPL) sequence and linking sequences were assembled as a block using PCR amplification (Figure 2). The ITR and encapsidation signal (sequences 1-392 of Ad-dl327 [identical to sequences from Ad5, Genbank accession #M73260] incorporated herein by reference) were amplified (amplification 1) together from Ad-dl327 using primers containing NotI or AscI restriction sites. The Rous Sarcoma Virus LTR promoter was amplified (amplification 2) from the plasmid pRC/RSV (sequences 209 to 605; Invitrogen, San Diego,

CA) using primers containing an AscI site and an SfiI site. DNA products from amplifications 1 and 2 were joined using the "overlap" PCR method (amplification 3) (Horton *t al.*, *BioTechniques*, *8*:528-535 (1990)) with only the NotI primer and the SfiI primer. Complementarity between the AscI-containing end of each initial DNA amplification product from reactions 1 and 2 allowed joining of these two pieces during amplification. Next the TPL was amplified (amplification 4) (sequences 6049 to 9730 of Ad-dl327 [identical to similar sequences from Ad5, Genbank accession #M73260]) from cDNA made from mRNA isolated from 293 cells (ATCC Accession No. CRL 1573) infected for 16 hrs. with Ad-dl327 using primers containing SfiI and XbaI sites respectively. DNA fragments from amplification reactions 3 and 4 were then joined using PCR (amplification 5) with the NotI and XbaI primers, thus creating the complete gene block.

Third, the ITR-encapsidation signal-TPL fragment was then purified, cleaved with NotI and XbaI and inserted into the NotI, XbaI cleaved PHR plasmid. This plasmid was designated pAvS6A' and the orientation was such that the NotI site of the fragment was next to the T7 RNA polymerase site (Figure 3).

Fourth, the SV40 early polyA signal was removed from SV40 DNA as an HpaI-BamHI fragment, treated with T4 DNA polymerase and inserted into the SalI site of the plasmid pAvS6a-(Figure 3) to create pAvS6 (Figures 3 and 4).

B. Construction of Av1Cf2 and Av1Luc1

Av1Cf2 (Figure 7) (Yei *et al.*, *Gene Therapy*, *1*:192-200 (1994)), an E1-deleted (1.18 map units to 9.2 map units), E3-deleted (78.5 map units to 84.7 map units) adenoviral vector constructed first by inserting the normal human CFTR cDNA coding sequence fragment into the EcoRV site of pAvS6 so that

the 5' end of the CFTR coding sequence was closest to the Adenovirus 5 tripartite leader. The CFTR cDNA was removed as a PstI fragment (nucleotides 75 to 4,725; for numbering see GenBank Accession No. M28688) from the plasmid pBQ4.7 (Figure 5) (provided by L.-C. Tsui, The Hospital for Sick Children, Toronto, Canada), and inserted as a blunt fragment. The resulting plasmid, pAvS6-CFTR (Figure 6) was linearized with KpnI and recombined with the large (35 kb) ClaI fragment of Ad-dl327 in 293 cells as described in Trapnell, *Advanced Drug Delivery Reviews, 12*:185-199 (1993) to form Av1Cf2 (Figure 7).

After double-plaque purification, the identify of the clonal isolates was confirmed by Southern analysis, immunoprecipitation of CFTR, as previously described. (Tolstoshev, *et al.*, Proceedings of the Ninth Nagoya International Symposium on Cancer Treatment, September 17-18, 1993, Nagoya, Japan (in press).

Av1Luc1 (Figure 7) (Yei *et al., Gene Therapy, Vol. 1, pgs. 192-200 (1994)*) is an adenoviral reporter vector identical in genomic organization and sequence to Av1Cf2, except that it expresses the firefly luciferase gene (Genbank Accession No. M15077).

The firefly luciferase gene was obtained from pGEM-luc (Figure 8 - Promega). pGEM-luc was digested with StuI and HindIII in order to splice out the firefly luciferase gene.

The firefly luciferase gene was inserted into the EcoRV site of pAvS6 so that the 5' end of the firefly luciferase coding sequence was closest to the Adenovirus 5 tripartite leader. The resulting plasmid, pAvS6-Luc1 (Figure 9) was linearized with KpnI and recombined with the large (35 kb) ClaI fragment of Ad-dl327 as hereinabove described.

Clonal isolates then were identified as hereinabove described.

Both viral vectors were propagated, purified by double-banding in CsCl gradients, and titered in 293 cells as described in Rosenfeld *et al.*, *Cell*, *68*:143-155 (1992).

Example 2

Adenoviral-mediated gene transfer with concurrent intermittent steroid administration

Cotton rats (weight approximately 150g) were divided into four groups with 9 rats in each group. Av1Cf2 was administered by intranasal inhalation (Yei *et al.*, *Human Gene Therapy*, Vol. 5, pgs. 731-744 (1994)) to the lungs of cotton rats at a low dose (10^8 pfu) or at a high dose (10^{10} pfu), either with or without coadministration of dexamethasone by intraperitoneal injection in an amount of 2 mg/kg daily, beginning 1 day prior to and continuing for 10 days after administration of the vector. A control group of rats was given PBS instead of Av1Cf2, either with or without coadministration of dexamethasone as hereinabove described.

At 3 days and at 42 days after vector administration, 3 rats from each group were evaluated for host responses to the Av1Cf2 vector. Evaluations of host responses included pulmonary histopathology appearance, total lung lavage cellularity, lung lavage anti-adenovirus antibody production, and cytotoxic T-lymphocyte (CTL) response.

Evaluation of pulmonary histopathology was performed as described in Yei *et al.*, *Human Gene Therapy*, Vol. 5, pgs 731-744 (1994). The lungs were removed, fixed in 2.5% (wt./vol.) paraformaldehyde, 0.25% (wt./vol.) glutaraldehyde at 4°C overnight, embedded in paraffin, and 6μm sections were stained with hematoxylin and eosin.

The histologic appearance of the lungs of the cotton rats in response to Av1Cf2 injection, with and without daily administration of immunosuppression therapy, at three days after adenoviral infection, is shown in Figures 10A, 10B, and 10C. Figure 10a is a section of the lung of a control rat that did not receive adenovirus and instead received PBS. Figure 10B is a section of the lung of a rat which received Av1Cf2 without immunosuppression therapy. Figure 10C is a section of the lung of a rat which received Av1Cf2 with daily administration of dexamethasone. All sections have been magnified 100 times. As shown in Figures 10A, 10B, and 10C, there was less pulmonary parenchymal inflammation in the rat which received adenovirus and immunosuppressive therapy as compared with the control rat and the rat infected with adenovirus, but did not receive immunosuppression therapy.

Lung lavage fluid was collected by lavaging the lung with 4.0 ml of PBS, and the total number of cells determined by counting in a hemocytometer, or the cells were evaluated in cytocentrifuge preparations for the percentage of neutrophils by light microscopy.

Figure 11A is a graph of the lung lavage cell count from rats infected with Av1Cf2 three days after infection, as compared with control rats which received PBS. The control rats either received dexamethasone or did not receive dexamethasone. Figure 11B is a graph of the lung lavage cell count from rats infected with Av1Cf2 at 42 days after infection, as compared with control rats which received PBS. The rats either received dexamethasone or did not receive immunosuppressant therapy.

As shown in Figure 11A, dexamethasone significantly reduced the non-specific host cellular inflammatory responses (represented by total lung lavage cellularity) at three days

after vector administration, which is the peak of inflammation.

Lung lavage anti-adenovirus production was measured by an ELISA assay carried out as follows.

10μl of Av1LacZ4 (Yei, *et al.*, Human Gene Therapy, Vol. 5, pgs. 731-744 (1994)) at $1x10^{11}$ pfu/ml was added to 90 μl of double distilled H_2O in a 0.5 ml Eppendorf tube. The tube was irradiated with ultraviolet light for 30 minutes in order to kill the adenovirus, and protein concentration was measured with a Bio-Rad kit. 8 ml of 0.1M Na_2CO_3 (pH 9.6) was added to the tube to provide a protein yield of 10 μg/4 ml or 125 ng of adenoviral antigen per 50 μl per well.

50μl of antigen then was added to each well of a 96-well microtiter plate (Immulon 2); and the plate was incubated at 37°C for 1 hour, or room temperature for 2 hours, or at 4°C overnight. The plate then was washed twice with PBS or double distilled H_2O.

300μl of blocking buffer (1% BSA in PBS) was added to each well, and the plate was incubated for 1 hour at room temperature. The plate then was rinsed with double distilled H_2O.

Blocking agent then was added to the background wells. 50μl of antibody samples (i.e., lung lavage samples prepared as hereinabove described) then were added to coated wells at serial two-fold dilutions, beginning at 1/4 and ending at 1/8192. 50 μl of negative control samples of serum from an uninfected cotton rat were added to another set of coated wells at the same serial dilutions. The plate the was incubated for 2 hours at room temperature, and 300 μl of 0.05% Tween 20/PBS then was added. The plate was incubated

for 5 minutes at room temperature, emptied, and 300μl of 0.05% Tween 20/PBS again was added. The plate again was incubated at room temperature for 5 minutes, and was emptied. The plate then was washed twice with double distilled H_2O or PBS.

Peroxidase-labeled goat anti-hamster IgG (10μg/10μl) was diluted with 10 ml BSA and PBS to make a working solution of 1 mg/ml concentration. (1:1,000 dilution). 100 μl of this solution then was added to each well, and the plate was incubated at room temperature for 2 hours. The plate then was washed five times with 300 μl of 0.01% Tween 20/PBS. The plate then was emptied and dried.

100 μl of tetramethyl benzidine (TMB) substrate was added to each well at room temperate, and the color was developed immediately. The blue color was monitored by reading at OD_{650} with an ELISA reader. The reaction was stopped when the OD_{650} was 0.5 to 0.6 by adding 100 μl of TMB stop solution to the wells. The OD_{450} then was read between 5 minutes and 1 hour after stopping the reaction. The antibody titer is the reciprocal of the dilution that gave an OD value of 0.1 larger than background OD. Alternatively, antibody titer also may be determined as 3 standard deviations above the OD of non-specific background. The average results, expressed in antibody titer, for the rats in each group at 3 days and 42 days after vector administration are shown in Figure 12. As shown in Figure 12, the rats which were given dexamethasone showed a decreased antibody titer 42 days after vector administration as compared with rats that were not given dexamethasone.

CTL assays were carried out at 42 days after vector administration.

Sensitizer cells were prepared by infecting cotton rat lung fibroblasts with Ad-dl327 at a multiplicity of infection of 100. The cells were incubated for 3 days, and checked for hexon expression by FACS. The cells then were washed with PBS/EDTA, contacted with trypsin, washed, spun, and resuspended in 1 ml RPMI medium. The cells then were irradiated with ^{137}Cs at 5,000 rads in order to inactivate the DNA.

Spleens then were isolated from uninfected (control) rats and adenovirus-infected rats 42 days after infection. The spleens were kept in sterile HBSS and ice. 10 ml of HBSS then was injected into each spleen with a 25/27 gauge needle. The spleen was mashed, and filtered with a cell strainer into a 50 ml tube. The volume then was brought to 40 ml in RPMI plus 10% FCS. The tube was spun at 1,500 rpm for 10 minutes. Red blood cells then were lysed by adding 2.5 ml of ACK lysis buffer, and the liquid was swirled for less than 1 minute. The volume was brought up to 50 ml with RPMI-10. The tube then was spun again at 1,500 rpm for 10 minutes. The cell pellet then was resuspended, and cells were counted at a 1:10 dilution. The splenocytes then were plated with the sensitizer cells at a ratio of splenocytes to sensitizer cells of 4:1 in RPMI. The splenocytes and sensitizer cells were incubated at 37°C in the presence of 20-50 units/ml of Interleukin-2. Interleukin-2 was added daily for 5 to 6 days.

Target cells were prepared by infecting $3X10^6$ cotton rat lung fibroblasts with Ad-dl327 at a multiplicity of infection of 100 for 1 hour. Culture medium is added to the cells, and ^{51}Cr in an amount greater than 50 μCi is added for 18 hours.

Target cells are harvested by washing the cotton rat lung fibroblasts with EDTA/PBS, followed by trypsinization.

The cells then were washed, spun, resuspended in 5 ml culture medium, and counted. The cells were resuspended to 10^5 c lls/ml and 10^4 cells/0.1 ml well were used for the CTL assay.

Effector cells (i.e., the combination of splenocytes and sensitizing cells (also sometimes referred to as Es cells) were spun at 1,500 rpm for 10 minutes at 4°C. The cells were resuspended in 2 ml of HBSS-10, loaded onto 7 ml Ficoll Hypaque, and spun at 1,500 rpm for 10 minutes. The top portion (4 ml) was harvested, and 5 ml of culture medium was added. This material was spun, the cell pellet was saved, and resuspended in 1 ml of culture medium. The effector cells were counted by mixing 50 µl of effector cells with 50 µl Trypan blue.

The effector cells then were added to wells containing 10^4 target cells, at effector:target (E:T) ratios of 3.125, 6.25, 12.5, 25, 50, and 100. The cells then were spun at 500 rpm for 5 minutes. The cells then were incubated at 37°C for 4 hours. The cells then were spun, and 100 µl of supernatant was analyzed for ^{51}Cr release with a WALLAC gamma counter. The average results for CTL response in splenocytes taken from infected rats (with and without dexamethasone treatment), and from two uninfected control rats, are shown in Figure 13.

As shown in Figure 13, a lower CTL response was obtained from splenocytes obtained from infected rats that were treated with dexamethasone.

At 42 days after vector administration, the remaining three rats in each group received an intranasal pulmonary administration of AvlLucl at a dose of $2x10^9$ pfu. Remaining control rats, which initially received PBS, also received

Av1Luc1 at a dose of $2x10^9$ pfu. Lung lavage anti-adenovirus antibody production was evaluated three days after administration of Av1Luc1, according to the procedure hereinabove described, and the results are shown in Figur 12. As shown in Figure 12, the rats which initially were given 10^{10} pfu of Av1Cf2, and were treated with dexamethasone, showed a decreased antibody titer as compared with rats that were not given dexamethasone.

The efficacy of transfer and expression of the firefly luciferase gene was evaluated three days after administration by quantifying directly intrapulmonary luciferase enzyme activity in light units (lu) by routine luminometry as described in Yei *et al., Gene Therapy, Vol. 1, pgs 192-200 (1994)*. The results are shown in Figure 14. As shown in Figure 14, each dot represents the luciferase enzyme activity for one rat, and the cross-bar represents the mean for each group. The efficiency of repeat adenovirus-mediated gene transfer was significantly higher in the rats which received Av1Cf2 and dexamethasone than those which did not receive dexamethasone at the time of the first adenoviral administration (11,786 ± 3523 lu vs. 622 ± 192 lu, respectively). The efficiency of gene transfer from Av1Luc1 also was higher in the control group which initially received PBS in conjunction with dexamethasone.

Example 3

Suppression of humoral immune response with DSG or high dose cyclophosphamide, permitting effective repeat administration of an adenoviral vector

This example describes the intravenous administration of the adenoviral vectors Av1LacZ4 and Av1H9F2 to C57BL/6 male mice (Harlan Sprague Dawley, Indianapolis, Indiana) at 5 to 6 weeks of age at the start of the experiment. Av1LacZ4 is an adenoviral vector which includes a nuclear targeted B-

galactosidase gene, (lacZ) and is described in PCT application No. W095/09654, published April 13, 1995. Av1H9F2 is constructed from a derivative of the adenoviral shuttle plasmid vector pAv1H9FR (Figure 15), which includes human Factor IX DNA, and is described in PCT application No. W094/29471, published December 22, 1994.

To construct Av1H9F2, the shuttle plasmid pAv1H9FR was digested with the restriction enzyme SfiI, the DNA ends were made blunt using T4 DNA Polymerase, and the DNA molecule was recircularized by ligation. Competent DH5 cells were transformed and ampicillin-resistant clones were amplified and screened by restriction enzyme digestion of miniprep DNA. A positive clone was identified and the resulting shuttle plasmid was referred to as pAvS17H9F.

Subsequently, 293 cells were cotransfected with pAvS17H9F and the large DNA fragment of ClaI digested Ad-dl327. Recombinant adenoviral vector plaques were picked, expanded, and screened for expression of Factor IX by ELISA. A positive clone was identified and amplified, thus generating the vector Av1H9F2. A schematic of the left end of the vector is shown in Figure 16. Av1H9F2 has a base pair deletion at the beginning of the tripartite leader, or TPL, which effectively changes the ATG into a CTG. The structure of the vector was verified by restriction enzyme diagnostics and by DNA sequence analysis of the region between the RSV promoter and the 3' untranslated region of the Factor IX cDNA.

Fifteen mice were immunosuppressed with 33 mg/kg of deoxyspergualin DSG (Nippon Kayaku Co. LTD, Tokyo, Japan), delivered intraperitoneally (ip), once daily, beginning the day before vector administration and continuing for a total of eight days. A vial containing 100 mg of lyophilized DSG

was reconstituted with 3.8 ml of injection grade water to yield a 25 mg/ml solution, which was aliquoted and frozen at -20°C. Each day, immediately before immunosuppression, an aliquot was thawed at room temperature and 0.7 ml was mixed with 6.3 ml of Hank's Balanced Salt Solution (HBSS) to yield a 2.5 mg/ml solution. The mice were weighed once, immediately prior to the first dose of DSG. Six mice received 1 x 10^8 pfu of Av1lacZ4 via tail vein injection on the second day of the immunosuppression regimen and 1 x 10^8 pfu of Av1H9F2 five weeks later. Another six mice received only Av1H9F2, five weeks after immunosuppression. Three mice were immunosuppressed, but received no adenoviral vector.

Six mice were immunosuppressed with a low dose (100 mg/kg) cyclophosphamide (Sigma) and fifteen mice were treated with a high dose (300 mg/kg). The animals received a single ip injection of cyclophosphamide one day before administration of adenoviral vector. All six mice which were treated with a low dose of cyclophosphamide also received 1 x 10^8 pfu of Av1lacZ4 the day after cyclophosphamide and 1 x 10^8 pfu of Av1H9F2 five weeks later. Six of the mice immunosuppressed with a high dose of cyclophosphamide received a 1 x 10^8 pfu of Av1lacZ4 the next day and 1 x 10^8 pfu of Av1H9F2 five weeks later. Another six did not receive Av1lacZ4 but did receive Av1H9F2. Finally, three mice were immunosuppressed, but received no adenoviral vector.

Twelve mice were immunosuppressed with 5 mg/kg dexamethasone (American Reagent Laboratories, Inc., Shirley, New York), delivered ip, once daily, beginning the day before vector administration and continuing for a total of eight days. Six of these mice receive 1 x 10^8 pfu of Av1lacZ4 on the second day of dexamethasone treatment and 1 x 10^8 pfu of Av1H9F2 five weeks later. Five immunosuppressed mice received only Av1H9F2 and one mouse received no vector.

Five weeks after administration of Av1lacZ4, but prior to administration of Av1H9F2, plasma was prepared from some mice and analyzed for antiadenovirus neutralizing antibodies. Neutralizing antibodies were detected in the plasma of mice which receive Av1LacZ4 without immunosuppression, however, mice which received vector and either DSG or high dose cyclophosphamide had no detectable neutralizing antibodies. In contrast, mice immunosuppressed with low dose cyclophosphamide or dexamethasone developed neutralizing antibodies.

Neutralizing antibody titers in 20 mice are given in Table I below. As indicated in Table I, DSG is deoxyspergualin, Cy is cyclophosphamide, and Dex is dexamethasone.

Table I

mouse	vector	immunosuppression	neutralizing Ab
1	none	none	0
2	none	none	0
3	$1x10^8$ pfu Av1lacZ4	none	64
4	$1x10^8$ pfu Av1lacZ4	none	32
5	$1x10^8$ pfu Av1lacZ4	none	32
6	$1x10^8$ pfu Av1lacZ4	none	32
7	none	33 mg/kg DSG	0
8	none	33 mg/kg DSG	0
9	$1x10^8$ pfu Av1lacZ4	33 mg/kg DSG	0
10	$1x10^8$ pfu Av1lacZ4	33 mg/kg DSG	0
11	none	300 mg/kg Cy	0
12	none	300 mg/kg Cy	0

13	$1x10^8$ pfu Av1lacZ4	100 mg/kg Cy	64
14	$1x10^8$ pfu Av1lacZ4	100 mg/kg Cy	8
15	$1x10^8$ pfu Av1lacZ4	300 mg/kg Cy	0
16	$1x10^8$ pfu Av1lacZ4	300 mg/kg Cy	0
17	none	5 mg/kg Dex	0
18	none	5 mg/kg Dex	0
19	$1x10^8$ pfu Av1lacZ4	5 mg/kg Dex	8
20	$1x10^8$ pfu Av1lacZ4	5 mg/kg Dex	8

One week after administration of Av1H9F2, plasma was prepared and analyzed by ELISA to determine the levels of human Factor IX. The results are shown in Figure 17. Mice which received Av1lacZ4 without immunosuppression, then received Av1H9F2 five weeks later, expressed no human Factor IX. Mice which received neither immunosuppression nor Av1lacZ4, but were treated with Av1H9F2, expressed an average of 9.2 μg/ml. Mice which were immunosuppressed with DSG at the time of Av1lacZ4 delivery, and then received Av1H9F2, expressed an average of 6.6 μg/ml. Mice which were immunosuppressed with DSG, but received no Av1lacZ4, and then treated with Av1H9F2, had an average level of 5.2 μg/ml. Finally, mice which were treated with DSG, but received neither vector, expressed no human Factor IX.

Mice immunosuppressed with a low dose of cyclophosphamide at the time of Av1lacZ4 administration, did not express human Factor IX after delivery of Av1H9F2. Mice treated with a high dose of cyclophosphamide, but not administered Av1lacZ4, expressed an average of 8 μg/ml one

week after delivery of Av1H9F2. Mice immunosuppressed with a high dose of cyclophosphamide at the time of Av1lacZ4 treatm nt, expressed an average of 15.4 μg/ml of human Factor IX after treatment with Av1H9F2. Mice treated with cyclophosphamide, but not treated with either adenoviral vector, did not express human Factor IX.

Mice immunosuppressed with dexamethasone, but not treated with Av1lacZ4, expressed an average of 5.5 μg/ml of human Factor IX one week after administration of Av1H9F2. However, mice which received Av1lacZ4 at the time of immunosuppression did not express Factor IX after delivery of Av1H9F2. Mice which were immunosuppressed, but not treated with either vector, did not express human Factor IX.

Example 4

Suppression of humoral immune response to adenoviral vectors to enable the repeat administrations thereof

This example is an elaboration and expansion of the data contained in Example 3. In this example, the following materials and methods were employed.

Adenoviral vectors

Av1LacZ4 and Av1H9F2 were described in Example 3 hereinabove. Av1H9FR was made by cotransfecting 293 cells with pAv1H9FR (Figure 15) with the large DNA fragment from ClaI digested Ad dl327. Recombinant adenoviral vector plaques were picked, expanded, and screened for expression of Factor IX by ELISA. A positive clone was identified and amplified, thus generating the vector Av1H9FR. This vector, like Av1H9F2, contains a centrally truncated first intron and the complete 5' and 3' untranslated regions from the human Factor IX gene. The centrally truncated first intron and 3' untranslated region are essentially the same sequences

described by Jallat, *et al.*, EMBO J., Vol. 9, pgs. 3295-3301 (1990).

Av1ALAPH81 is an adenoviral vector which contains the B-domain deleted human Factor VIII cDNA expressed from the mouse albumin promoter, and is described in published PCT Application No. WO94/29471.

All vector stocks contained less than 1 in 10^6 wild-type adenovirus, as determined by quantitative PCR analysis of E1a sequences.

Immunosuppressants

Deoxyspergualin (manufactured by Nippon Kayaku Co., Ltd., Tokyo, Japan) was a gift from Bristol-Myers-Squibb, Princeton, N.J. A 100 mg vial of deoxyspergualin was reconstituted with water to a final concentration of 25 mg/ml, aliquoted, and frozen at -70°C. Frozen stocks were thawed at room temperature and diluted with Hanks Balanced Salt Solution (HBSS) prior to injection.

Deoxyspergualin is an immunosuppressant currently being tested clinically in organ transplantation. It has a potent, long term effect on antigen specific B cells and has been shown to prevent effectively the production of specific antibody when co-administered with protein antigens. (Alegre, *et al.*, Transplantation, Vol. 57, pgs. 1786-1794 (1994); Tepper, Ann. N. Y. Acad. Sci., pgs. 123-132 (1993); Tufveson, *et al.*, Transplant. Proc., Vol. 26, pgs. 3029-3039 (1994)). The mode of action of DSG is not fully understood at the molecular level. The data suggest that it may interfere with differentiation of B and T cells and also with antigen processing. Recent studies showed that DSG inhibited κ light chain expression and therefore blocked IgM expression on the surface of pre B cells. (Tepper, 1993). In addition,

the data showed that DSG inhibited nuclear translocation of NFκB (Tepp r, 1993), which could be the mechanism by which DSG inhibits differentiation of B and T cells. Finally, it has been demonstrated that DSG binds to Hsc70, a heat shock protein. (Tepper, 1993). Heat shock proteins are involved in protein folding, molecular chaperoning, peptide loading of MHC molecules, and antigen presentation. Therefore, binding of DSG to Hsc70 may explain the effect on antigen presentation.

Cyclophosphamide (Cy) was obtained from Sigma and Dissolved in HBSS. Dexamethasone (Dex) solution from American Regent Laboratories, Inc., Shirley, N. Y. was diluted in HBSS prior to injection. All three immunosuppressants were delivered intraperitoneally, according to the doses indicated hereinbelow.

Animal procedures

C57BL/6 mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). Adenoviral vectors were administered via tail vein injection after diluting the appropriate amount of vector stock to 0.5 ml with Hanks Balanced Salt Solution (HBSS). At the time points indicated in the text, blood was obtained from the retroorbital plexus. For preparation of plasma samples, sodium citrate was added immediately to a final concentration of 0.38% (w/v). To prepare sera samples, the blood was allowed to clot. Samples were centrifuged for 5 min. in an Eppendorf Microfuge after which the plasma or serum was collected, aliquoted, and frozen.

Human Factor VIII ELISA

Plasma levels of human Factor VIII were determined by ELISA, as described in Connelly, *et al.*, Human Gene Therapy, Vol. 6, pgs. 185-193 (1995). The limit of sensitivity with mouse plasma samples containing B domain deleted human Factor

VIII was 3 to 6 ng/ml. Mouse plasma samples were diluted 1:5 prior to the assay, ther fore, the actual limit of detection was 15 to 30 ng/ml.

Human Factor IX ELISA

Plasma levels of human Factor IX were determined by ELISA. Asserachrom IX:Ag ELISA kits were purchased from American Bioproducts Company (Parsippany, NJ) and assays were performed according to the manufacturer's instructions. The limit of sensitivity was 1.6 ng/ml.

Anti-adenoviral antibody assay

Mouse plasma or serum samples were heat inactivated at 55°C for 30 minutes and then diluted in Improved Minimal Essential Medium (Biofluids, Rockville, MD) plus 2% FBS (IMEM/2%FBS) in two-fold steps beginning at 1:2. 55 μl of each sample were mixed with 10 μl of Av1lacZ4 (containing 4 x 10^5 pfu), incubated for 1 hour at 37°C and applied to nearly confluent 293 cells in 96 well plates (4 x 10^4 cells per well). After 60 minutes in the tissue culture incubator, the virus was aspirated from each well and replaced with 150 μl of IMEM/10%FBS. The following day, cells were fixed and stained for β-galactosidase expression, as described previously. (Smith, *et al.*, Nature Genetics, Vol. 5, pgs. 397-402 (1993)). In the absence of inactivating antibodies, all of the cells stained blue. The titer of inactivating antibodies for each sample was reported as the reciprocal of the highest dilution with which less than 25% of the cells stained blue.

Results

Dose dependence of the humoral immune response to adenovirus vectors

Previous studies which have examined repeat delivery of adenovirus vectors have employed relatively high doses of

vector, which would be expected to maximize the strength of the immune response. (Smith, *et al.*, 1993; Kozarsky, *et al.*, J. Biol. Chem., Vol. 269, pgs. 13695-13702 (1994); Kay, *et al.*, Proc. Nat. Acad. Sci., Vol. 91, pgs. 2353-233357 (1994); Yei, *et al.*, Gene Therapy, Vol. 1, pgs. 192-200 (1994); Yang, *et al.*, J. Virol., Vol. 69, pgs. 2004-2015 (1995); Dai, *et al.*, Proc. Nat. Acad. Sci., Vol. 92, pgs. 1401-1405 (1995); Barr, *et al.*, Gene Therapy, Vol. 2, pgs. 151-155 (1995)).

To determine whether the production of neutralizing antibodies and the block to repeat delivery is dependent on the dose of vector inoculated, various doses of the adenovirus vector Av1lacZ4 to C57BL/6 mice via tail vein were administered. The vector inoculum ranged from 1 x 10^3 pfu to 1 x 10^8 pfu in single log increments. Thirty-four days after vector delivery, the serum levels of anti-adenovirus neutralizing antibodies were determined (Fig. 18) for mice which received 1x10^5 pfu or greater of vector. A minus sign indicates that none of the mice in the cohort had detectable antibody. The plus sign corresponding to mice which received 1x10^8 pfu of vector indicates that three of the five mice had an antibody titer of 8, while two mice had no detectable antibody. Thus, three of five mice which received 1 x 10^8 pfu had a level of anti-adenovirus antibody which was sufficient to neutralize 4x10^5 pfu of Av1LacZ4, while two mice had undetectable levels. None of the mice which received lower doses of vector had detectable antibodies using this relatively stringent neutralization assay.

Thirty-five days after administration of Av1lacZ4, each mouse received 2 x 10^8 pfu of Av1H9FR, an adenoviral vector encoding human Factor IX. One week later, the plasma levels of human Factor IX were determined by ELISA (Fig. 18). An average of approximately 2 μg/ml of Factor IX was detected in mice which received either no Av1lacZ4, or up to 1 x 10^5 pfu

of the first vector. Factor IX was also readily detected in the mice which had received a first dose of 1 x 10^6 and 1 x 10^7 pfu, although the levels were reduced. Mice which received 1 x 10^8 pfu of Av1lacZ4 yielded little or no human Factor IX after administration of Av1H9FR. Thus effective gene transfer and expression can be achieved with a second vector administration, provided the initial vector dose is below a certain threshold level. The data indicate that for intravenous delivery in C57BL/6 mice, this value is between 10^7 and 10^8 pfu.

Transient immunosuppression increases the efficiency of vector re-administration.

To determine whether transient immunosuppression would allow readministration of an adenoviral vector, C57BL/6 mice were immunosuppressed with either deoxyspergualin (DSG), cyclophosphamide (Cy), or dexamethasone (Dex) at the time of administration of 1 x 10^8 pfu of Av1lacZ4. As shown above, this dose completely prevented an effective second delivery. Mice were injected daily with 33 mg/kg of DSG, beginning one day before vector delivery and continuing for seven more days. Dexamethasone was delivered over the same time course, at a dose of 5 mg/kg. Cyclophosphamide was administered once, the day before vector delivery, at a dose of either 100 mg/kg or 300 mg/kg. Control mice received Av1lacZ4 without immunosuppression, or were immunosuppressed without initial vector delivery.

Five weeks after vector administration, the plasma levels of anti-adenovirus neutralizing antibodies were determined (Fig. 19). Mice immunosuppressed with DSG or 300 mg/kg of cyclophosphamide had no detectable neutralizing antibodies, but all other mice which received Av1lacZ4 generated neutralizing antibodies. Thirty-five days after the first vector injection, the mice received 1 x 10^8 pfu of

Av1H9F2. One week after Av1H9F2 injection, plasma samples were prepared and the levels of human Factor IX were determined by ELISA (Fig. 17). Mice immunosuppressed with DSG or 300 mg/kg cyclophosphamide expressed human Factor IX at levels which were approximately the same as levels in mice which had not been treated with the first vector. No human Factor IX was detected in the plasma of the mice which were not immunosuppressed at the time of Av1lacZ4 administration. Under the conditions used in these studies, immunosuppression with dexamethasone or 100 mg/kg cyclophosphamide was not effective in permitting expression of Factor IX on repeat injection. Thus the ability to achieve transgene expression on a second vector injection correlated with the suppression of neutralizing antibodies by DSG or high dose cyclophosphamide treatment.

At the time of administration of the second vector, Av1H9F2, half of the mice in each cohort which had been immunosuppressed with cyclophosphamide or dexamethasone were immunosuppressed again, using the same regimen as at the first vector delivery. Five weeks later, plasma levels of anti-adenovirus neutralizing antibodies were determined. The mice immunosuppressed with 300 mg/kg cyclophosphamide had no detectable neutralizing antibodies, while mice immunosuppressed with 100 mg/kg cyclophosphamide or 5 mg/kg dexamethasone had a measurable response (data not shown). Thirty-five days after Av1H9F2 injection, 1 x 10^9 pfu of a Factor VIII vector, Av1ALAPH81, were administered to the mice which had been immunosuppressed with 300 mg/kg cyclophosphamide. In addition, Factor VIII vector was administered to control mice which had received Av1lacZ4 and Av1H9F2 without immunosuppression and also to naive mice. One week later, plasma levels of human Factor VIII were determined by ELISA (Fig. 20). Control mice which received only the Av1ALAPH81 vector, and mice immunosuppressed with

300 mg/kg of cyclophosphamide at the time of the two prior vector injections expressed human Factor VIII. Mice which received Av1lacZ4 and Av1H9F2 without imunosuppressi n, as well as mice which were immunosuppress d only at the time of Av1lacZ4 delivery, did not express human Factor VIII.

DSG permits effective repeat administration at a clinically relevant dose.

The previous experiment demonstrated that a high dose of either DSG or cyclophosphamide permitted readministration of an adenoviral vector, but that clinically relevant doses of cyclophosphamide and dexamethasone were not effective. The next objective was to determine whether lower doses of DSG would be effective. The dose of DSG used in the initial experiment (33 mg/kg) is near the maximum tolerated dose in mice and is significantly higher than the 5-7 mg/kg dose used in human clinical trials for organ transplantion. (Suzuki, *et al.*, Ann. N.Y. Acad. Sci., Vol. 696, pgs. 263-269 (1993); Jindal, *et al.*, Mt. Sinai J. Med., Vol. 61, pgs. 51-56 (1994)). To determine if lower doses would also be effective in allowing vector readministration, mice were immunosuppressed with 5, 10, 20, and 33 mg/kg of DSG at the time of administration of 1 x 10^9 pfu of Av1lacZ4. Immunosuppression was started the day before vector delivery and continued for a total of 8 days. On the day of vector delivery, DSG was given after injection of the adenovirus since it is most effective when administered after antigen. (Takahara, *et al.*, Transplantation, Vol. 53, pgs. 914-918 (1992)).

Thirty-five days later each mouse received 1 x 10^9 pfu of Av1H9F2. One week after Av1H9F2 injection, human Factor IX plasma levels were determined by ELISA (Fig. 21). Control mice, which were not pre-immunized with Av1LacZ4, expressed an average of 9 μg/ml of human Factor IX. Other control

mice, which received Av1lacZ4 but were not immunosuppressed, expressed no human Factor IX after Av1H9F2 administration. The one mouse which was immunosuppressed with 33 mg/kg DSG expressed 12 μg/ml of human Factor IX. Five of six mice immunosuppressed with 20 mg/kg DSG expressed an average of 3.0 μg/ml of human Factor IX, and one mouse expressed none. Mice immunosuppressed with a dose of 10 mg/kg expressed a wide range of Factor IX, extending from 30 ng/ml to 8 μg/ml. Three mice immunosuppressed with 5 mg/kg expressed no human Factor IX, while three others expressed levels ranging from 2 to 7 μg/ml. Mice which were not immunosuppressed at the time of Av1lacZ4 administration expressed no human Factor IX.

Discussion

The data demonstrate that multiple intravenous administrations of adenovirus vectors with resulting transgene expression can be accomplished in immune competent animals treated with a short course of immunosuppression at the time of vector delivery. This observation is significant because several recent studies have demonstrated that a humoral immune response directed against adenoviral vector prevents readministration. (Smith, *et al.*, 1993; Kay, *et al.*, 1994; Yei, *et al.*, 1994; Yang, *et al.*, 1995.) The inability to readminister vector has presented a major obstacle to the clinical utility of adenoviral vectors, since effective readministration will almost certainly be required for the clinical application of these vectors to gene therapy of chronic diseases.

The failure to obtain expression following repeat dosing in the absence of immunosuppression correlates with anti-adenovirus neutralizing antibodies. Evidence that such antibodies are sufficient to block readministration was provided by Yang *et al.*, 1995, who showed that passive transfer of serum from a mouse previously treated with vector

into the venous circulation of a naive mouse was able to prevent vector-mediated gene expression in the liver. The role of the immune system in preventing repeat administration also was demonstrated by the observation that repeat administration of vector was effective in immunodeficient mice. (Yang, *et al.*, 1995; Dai, *et al.*, 1995; Barr, *et al.*, Gene Therapy, Vol. 2, pgs. 151-155 (1995)). This result has been confirmed by demonstrating effective administration of Av1H9F2 to scid mice 5 weeks after delivery of 5 x 10^8 pfu of Av1ALAPH81 (data not shown).

Since previous reports describing humoral responses to adenovirus administration used relatively high vector doses, we evaluated the relationship between initial vector dose and the ability to achieve an effective repeat gene transfer. The results indicated that the magnitude of the immune response was dependent on the initial dose of vector and that if the dose is below a threshold level, a second administration is possible. The finding that this level was only one to two orders of magnitude below a clinically relevant vector dose further suggested that immunosuppression at the time of vector delivery would permit readministration. In addition, these results emphasize the need for potent vectors which would be efficacious at low doses.

Applicants have observed that anti-adenovirus neutralizing antibody titers are maintained for at least ten months in mice after a single administration of vector via tail vein. The long-term maintenance of titer may have been due to a low level of ongoing adenoviral backbone gene expression in transduced cells. Vectors designed to reduce or eliminate backbone gene expression may elicit a weaker immune response and therefore may require less immunosuppression for successful readministration.

An important property of DSG is that it does not produce a general suppr ssion of the immune system, but rather results in a selective lack of response to sp cific antig ns pr sented at the time of drug treatment. We found that immunosuppression with DSG over a 7 day period following vector delivery efficiently inhibited the humoral response to the vector and permitted an effective second administration. The initial experiment with DSG employed a high dose of 33 mg/kg, which is close to the maximum tolerated dose in mice and several fold higher than the doses used in human trials. When administered over the same 8 day course, including 7 days post vector treatment, lower doses of DSG were also effective in permitting repeat delivery of vector. A greater degree of individual variability in levels of Factor IX expression was seen with reduced doses, although even at the lowest dose tested (5 mg/kg) significant Factor IX expression was obtained in 3 of 6 animals.

Cyclophosphamide, administered at a dose of 300 mg/kg the day before vector injection, was also effective in blocking the humoral response and allowed a completely effective second injection with a Factor IX adenovirus vector. Furthermore, a third injection with a Factor VIII encoding vector was also completely efficacious when the previous two vector administrations were each preceded by a single dose of cyclophosphamide. Cyclophosphamide is used clinically as an anti-cancer agent in the treatment of Hodgkins disease and other leukemias. It is also employed as an immunosuppressive agent in the treatment of hemophilia patients who develop inhibitors to Factor VIII protein replacement therapy. (Aledort, Am. J. Hemat., Vol. 47, pgs. 208-217 (1994); Nilsson, *et al.*, N. Engl. J. Med., Vol. 318, pgs. 947-950 (1988)). While the dose used to successfully obtain readministration in mice is substantially higher than is generally used in humans, it remains to be established

whether lower, clinically acceptable doses, might be effective in humans. One possibility suggested by experience in the organ transplantation setting is that combinations of immunosuppressants would yield more potent suppression of the immune system with less toxicity. For example, cyclophosphamide may be effective at lower doses when used in combination with dexamethasone. It is also possible that the degree of immunosuppression required will depend on the dose of vector which is needed to effect therapy. The dose of Av1H9F2 used in this study, 1 x 10^8 pfu, yielded plasma levels of human Factor IX of 5-10 μg/ml, which is 20 to 50 times above a level that would be therapeutic in a hemophiliac. Vectors, such as Av1H9F2, which express high levels of transgene product and which can be administered at relatively low doses, should reduce the extent of immune stimulation and the degree of immunosuppression required.

In summary, Applicants have shown that effective repetitive delivery of systemically administered adenovirus vectors can be achieved with short term immunosuppression. Importantly, this can be accomplished using pharmacologic agents which are either approved for use in humans, or are in clinical testing.

The disclosure of all patents, publications, (including published patent applications), and database accession numbers, and depository accession numbers referenced in this specification are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication, and database accession number, and depository accession number were specifically and individually indicated to be incorporated by reference.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific

embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

WHAT IS CLAIMED IS:

1. A method of effecting a gene therapy treatment in a host, comprising the steps of:

(a) administering to a host (i) an adenoviral vector including at least one DNA sequence encoding a therapeutic agent and (ii) an immunosuppressive agent;

(b) discontinuing said administration of said adenoviral vector and said immunosuppressive agent; and

(c) repeating the course of administration of said adenoviral vector including at least one DNA sequence encoding a therapeutic agent, and said immunosuppressive agent of step (a) at least once, said adenoviral vector being administered in an amount effective to produce a therapeutic effect in said host, and said immunosuppressive agent being administered in an amount effective to prevent or suppress an immune response against said adenoviral vector in said host.

2. The method of Claim 1 wherein said immunosuppressive agent is a steroid.

3. The method of Claim 2 wherein said steroid is dexamethasone.

4. The method of Claim 1 wherein said immunosuppressive agent is cyclosporin A.

5. The method of claim 1 wherein said adenoviral vector is administered each time in an amount of from about 1 pfu to about 10^{-4} pfu.

6. The method of Claim 3 wherein said dexamethasone is administered each time in an amount of from about 1 mg/kg to about 15 mg/kg.

7. The method of Claim 6 wherein said dexamethasone is administered each time in an amount of about 2 mg/kg.

8. The method of Claim 5 wherein said adenoviral vector is administered each time in an amount of from about 10^6 pfu to about 10^{13} pfu.

9. The method of Claim 8 wherein said adenoviral vector is administered each time in an amount of from about 10^8 pfu to about 10^{10} pfu.

10. The method of Claim 1 wherein said immunosuppressive agent is deoxyspergualin.

11. The method of Claim 10 wherein said deoxyspergualin is administered each time in an amount of from about 1 mg/kg to about 33 mg/kg.

12. The method of Claim 1 wherein said immunosuppressive agent is cyclophosphamide.

13. The method of Claim 12 wherein said cyclophosphamide is administered each time in an amount of from about 5 mg/kg to about 300 mg/kg.

14. The method of Claim 1 wherein said deoxyspergualin is administered each time in an amount of from about 3 mg/kg to about 7 mg/kg.

15. The method of Claim 13 wherein said cyclophosphamide is administered each time in an amount of from about 50 mg/kg to about 100 mg/kg.

16. The method of Claim 1 wherein said immunosuppressive agent is administered for a period f time which does not exceed 14 days.

17. The method of Claim 16 wherein said immunosuppressive agent is administered for a period of time which does not exceed 11 days.

18. The method of Claim 17 wherein said immunosuppressive agent is administered for a period of time which does not exceed 8 days.

19. The method of Claim 1 wherein said administration of said immunosuppressive agent is begun at about 24 hours prior to administration of said adenoviral vector.

20. The method of Claim 1 wherein said administration of said immunosuppressive agent is begun at the same time as administration of said adenoviral vector.

21. The method of Claim 1 wherein said administration of said immunosuppressive agent is begun at about 24 hours after administration of said adenoviral vector.

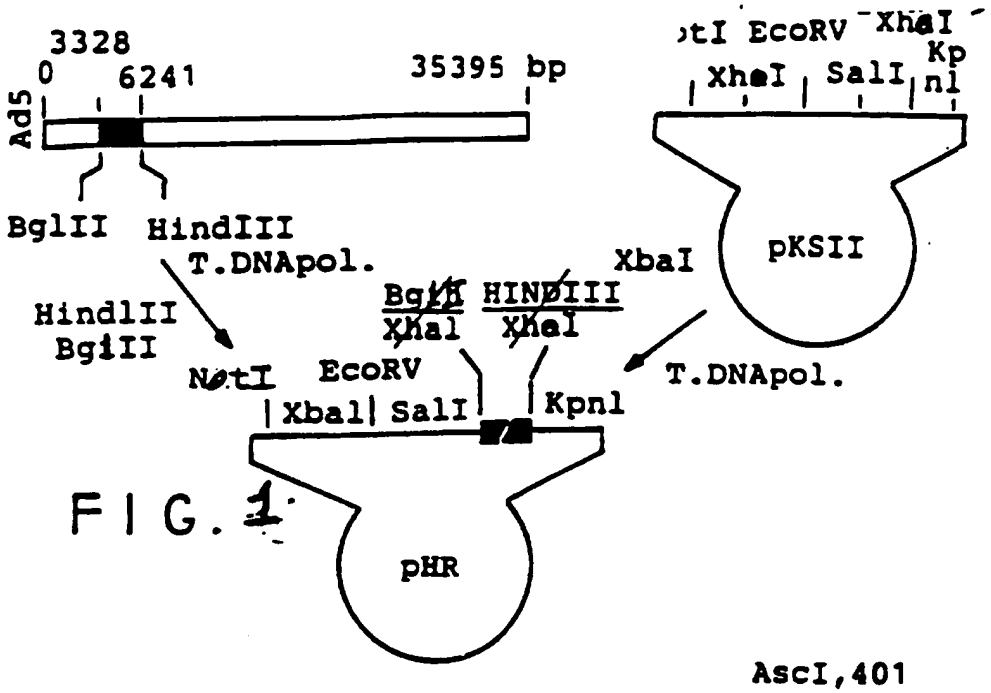

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FIG. 1

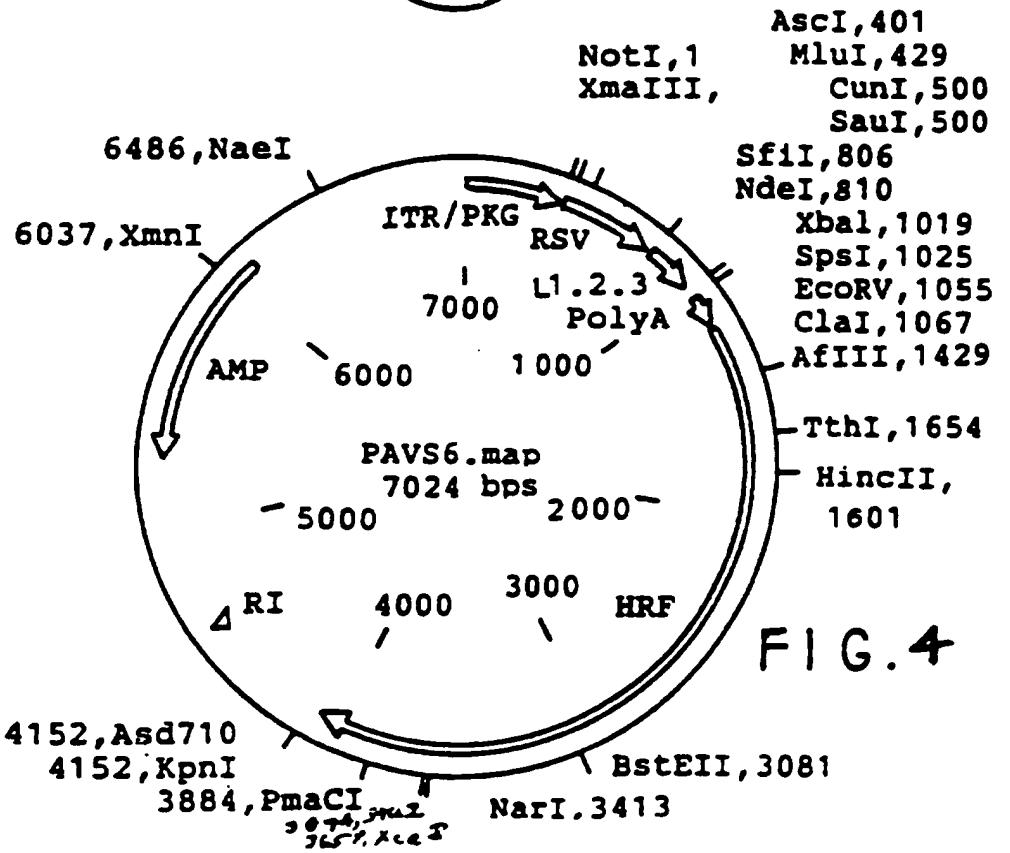
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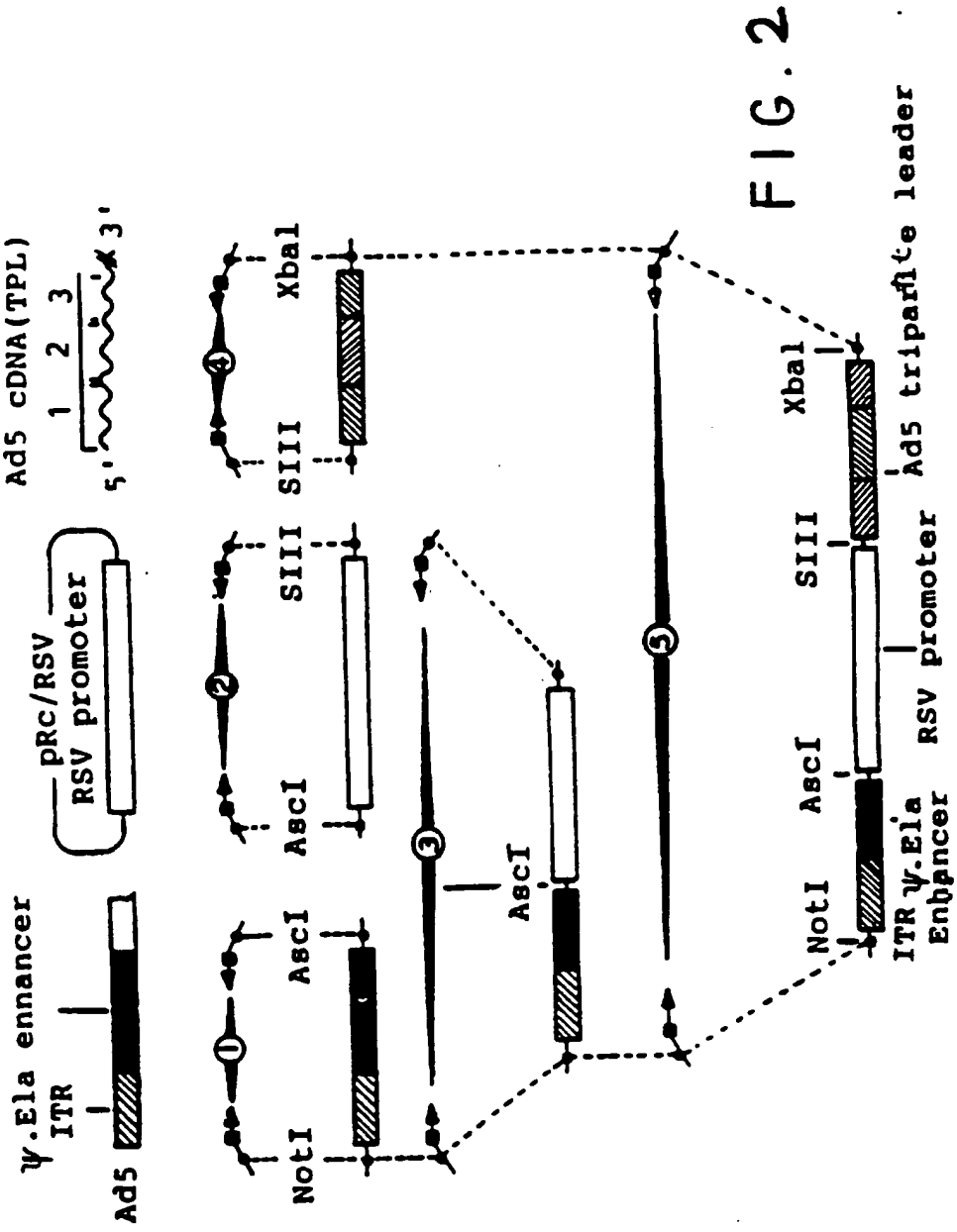
FIG. 4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Ad5
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RSV promoter
Ad5 cDNA(TPL)
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AscI
SIII
Xbal
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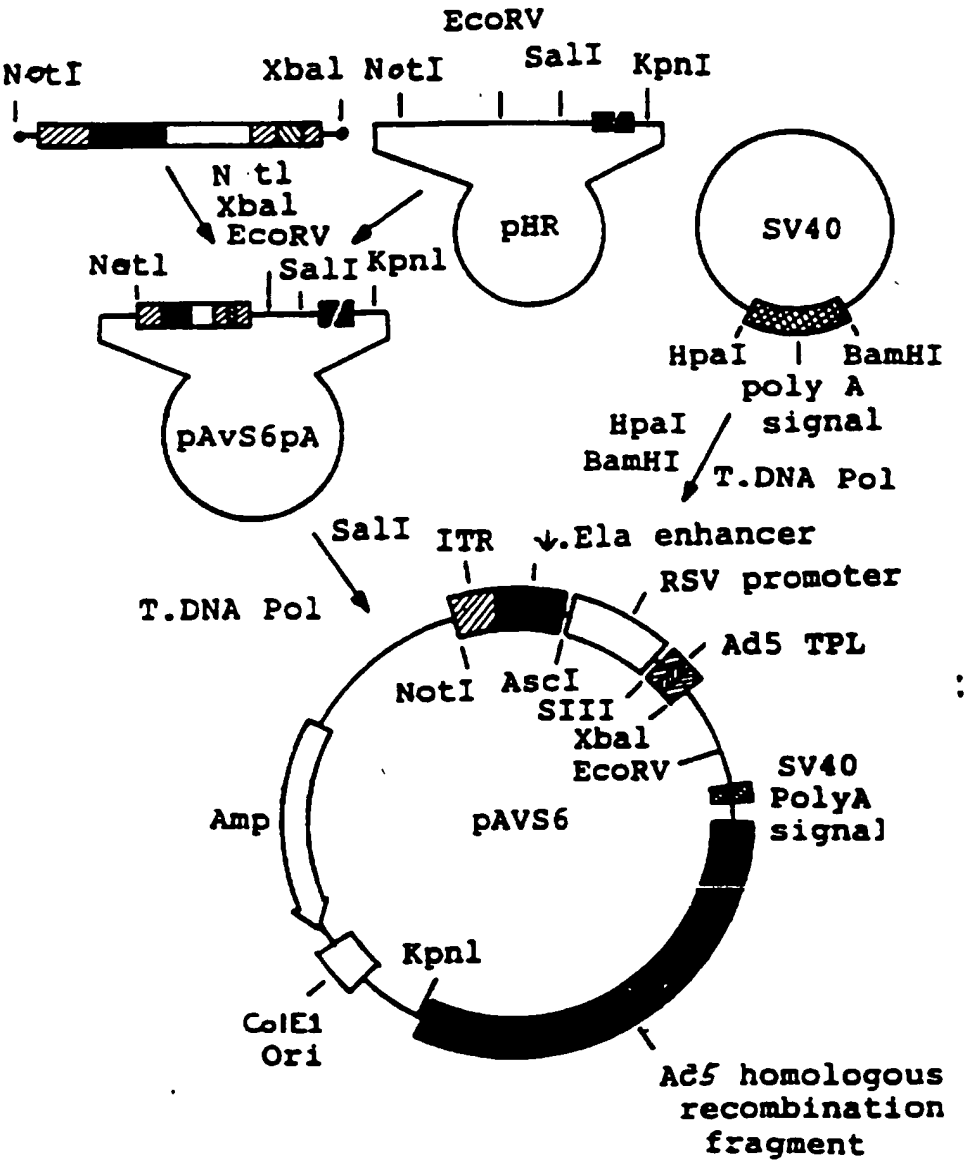
RSV promoter
Ad5 tripartite leader

FIG. 2

FIG. 3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poly A
signal
HpaI
BamHI
T.DNA Pol
SalI
ITR
Ela enhancer
RSV promoter
T.DNA Pol
Ad5 TPL
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AscI
SIII
XbaI
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Ad5 homologous
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fragment

Fig. 5:

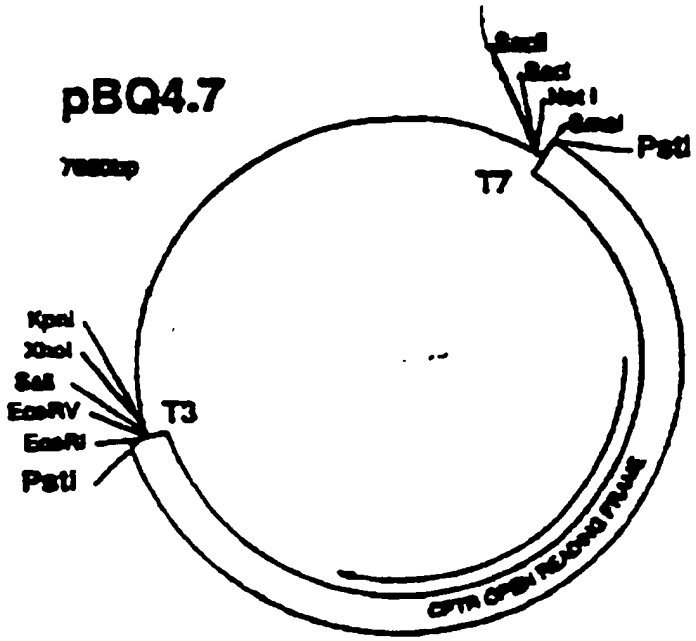
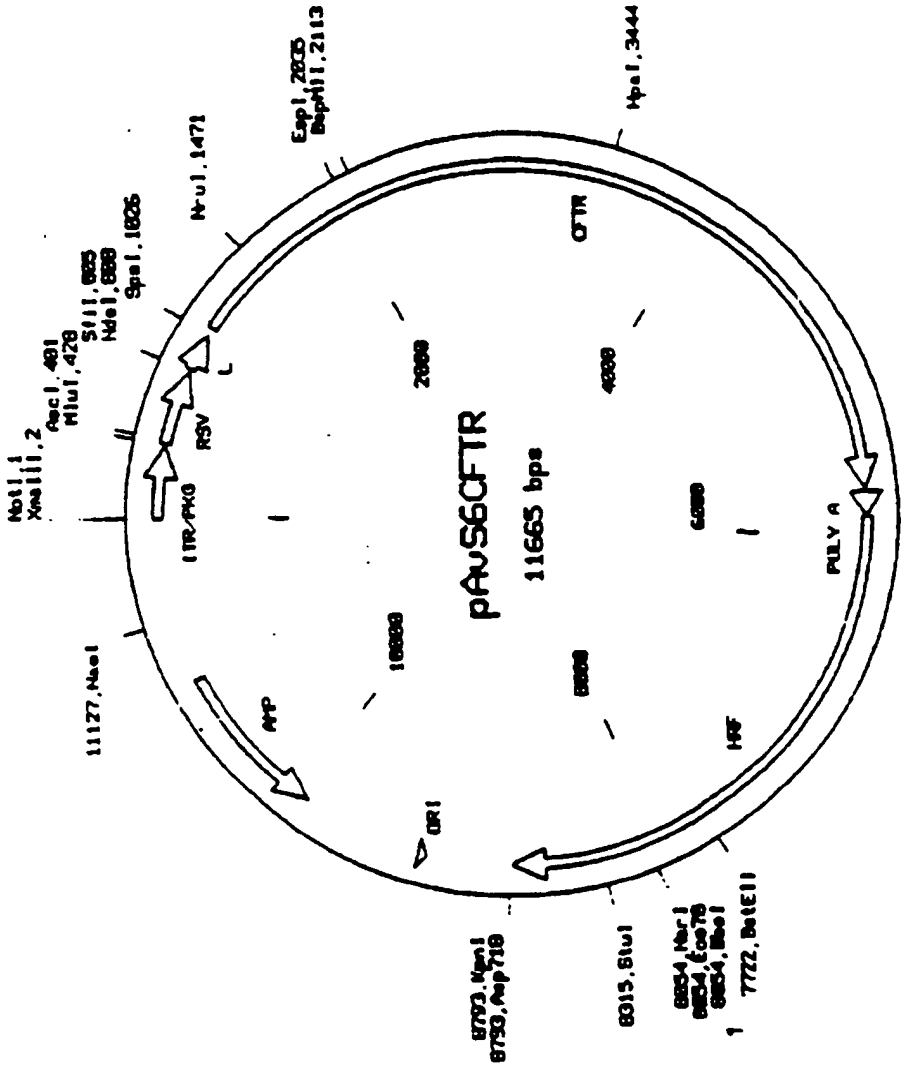


Fig. 5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MluI.428
SfiI.685
NdeI.888
SpeI.1806
NruI.1471
EspI.2035
BspHII.2113
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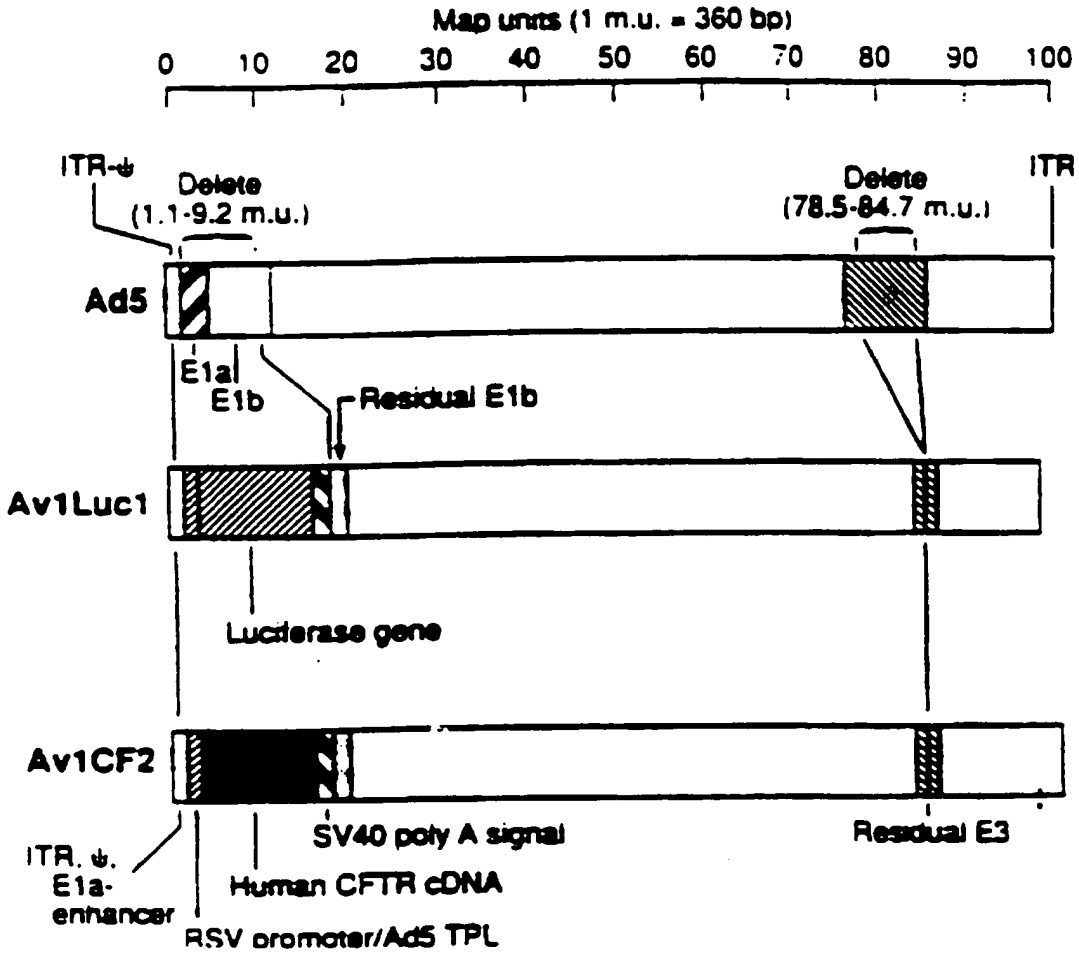
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E1b
Residual E1b
Av1Luc1
Luciferase gene
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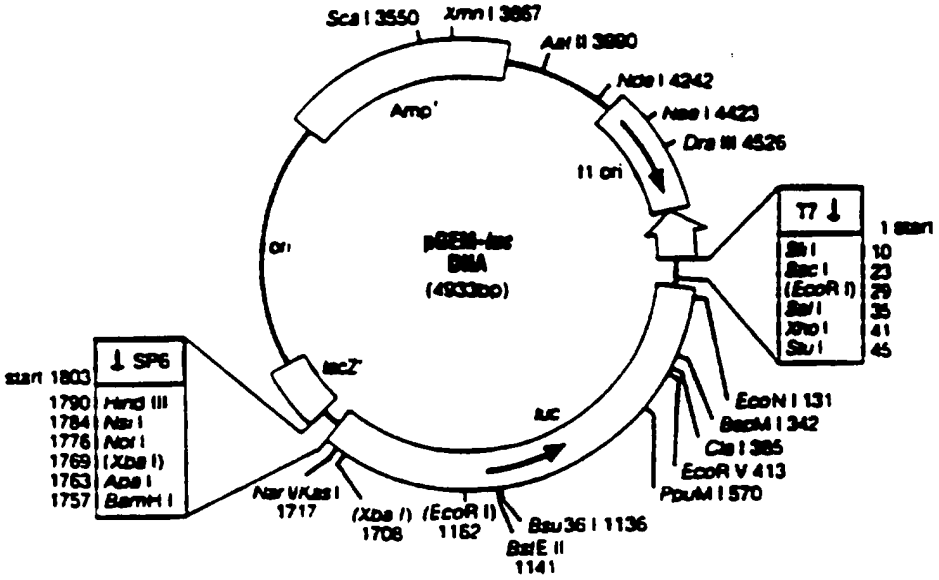
E1a-
enhancer
Human CFTR cDNA
RSV promoter/Ad5 TPL

Fig. 7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lacZ'
luc
T7
1 start
Sfi I 10
Sac I 23
(EcoR I) 29
Sal I 35
Xho I 41
Stu I 45
EcoN I 131
BsaM I 342
Cla I 385
EcoR V 413
PpuM I 570
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(Xba I) 1708
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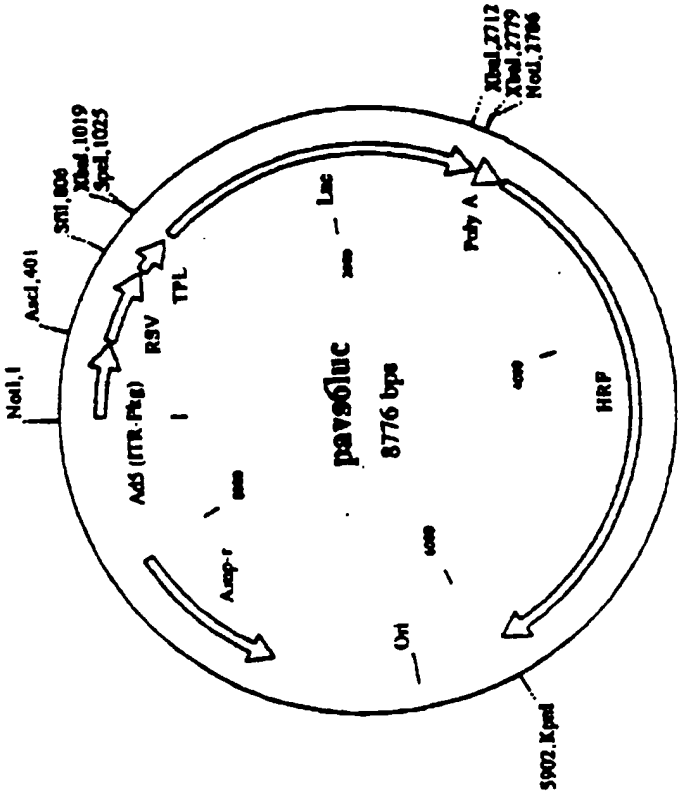
Fig. 8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Luc
Poly A
HRP
Ori
Amp-r
Ad5 (ITR.Pkg)
RSV
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NotI.1
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SfiI.806
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Fig. 9

Fig. 10

13253

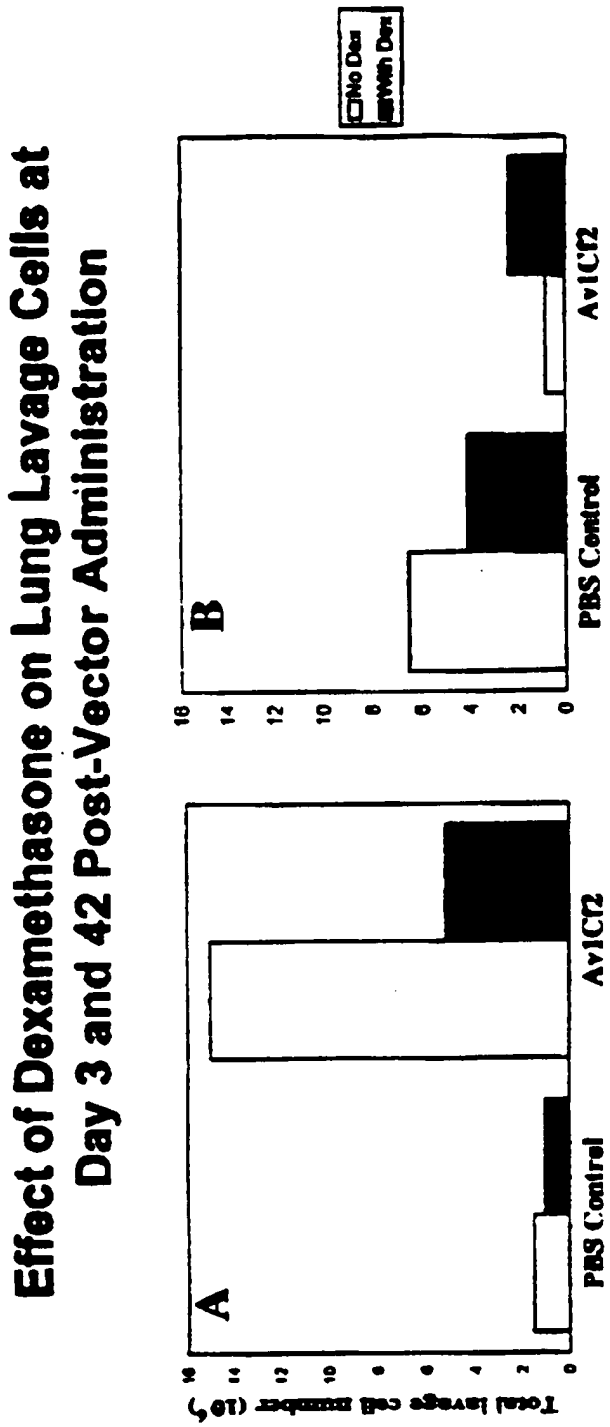

Effect of Dexamethasone on Lung Lavage Cells at Day 3 and 42 Post-Vector Administration
A
B
No Dex
With Dex
PBS Control
Av1Ct2
Total lavage cell number (10⁶)
0
2
4
6
8
10
12
14
16

Fig. 11

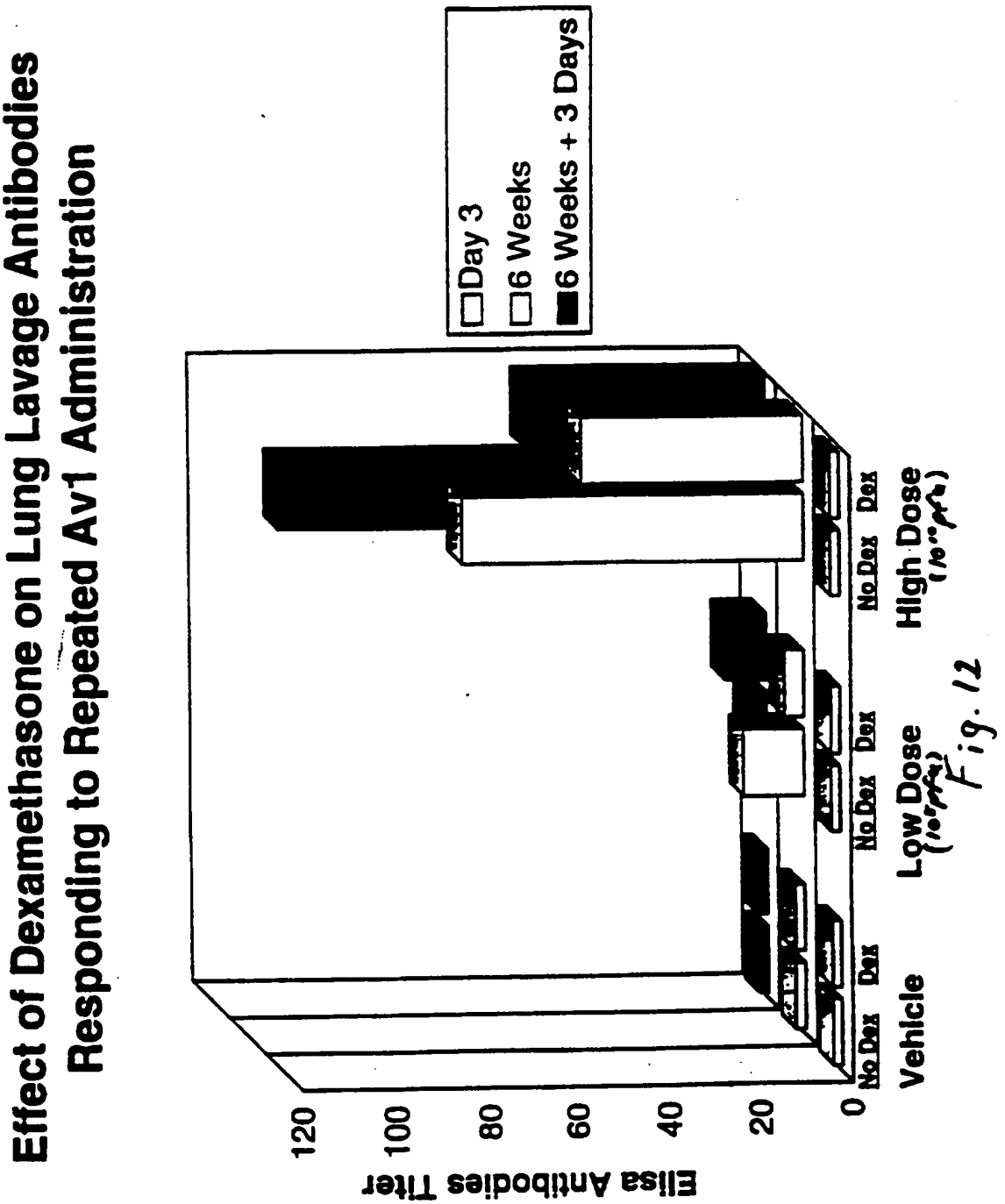

Effect of Dexamethasone on Lung Lavage Antibodies
Responding to Repeated Av1 Administration
Day 3
6 Weeks
6 Weeks + 3 Days
Elisa Antibodies Titer
120
100
80
60
40
20
0
No Dex
Dex
Vehicle
Low Dose
High Dose

Fig. 12

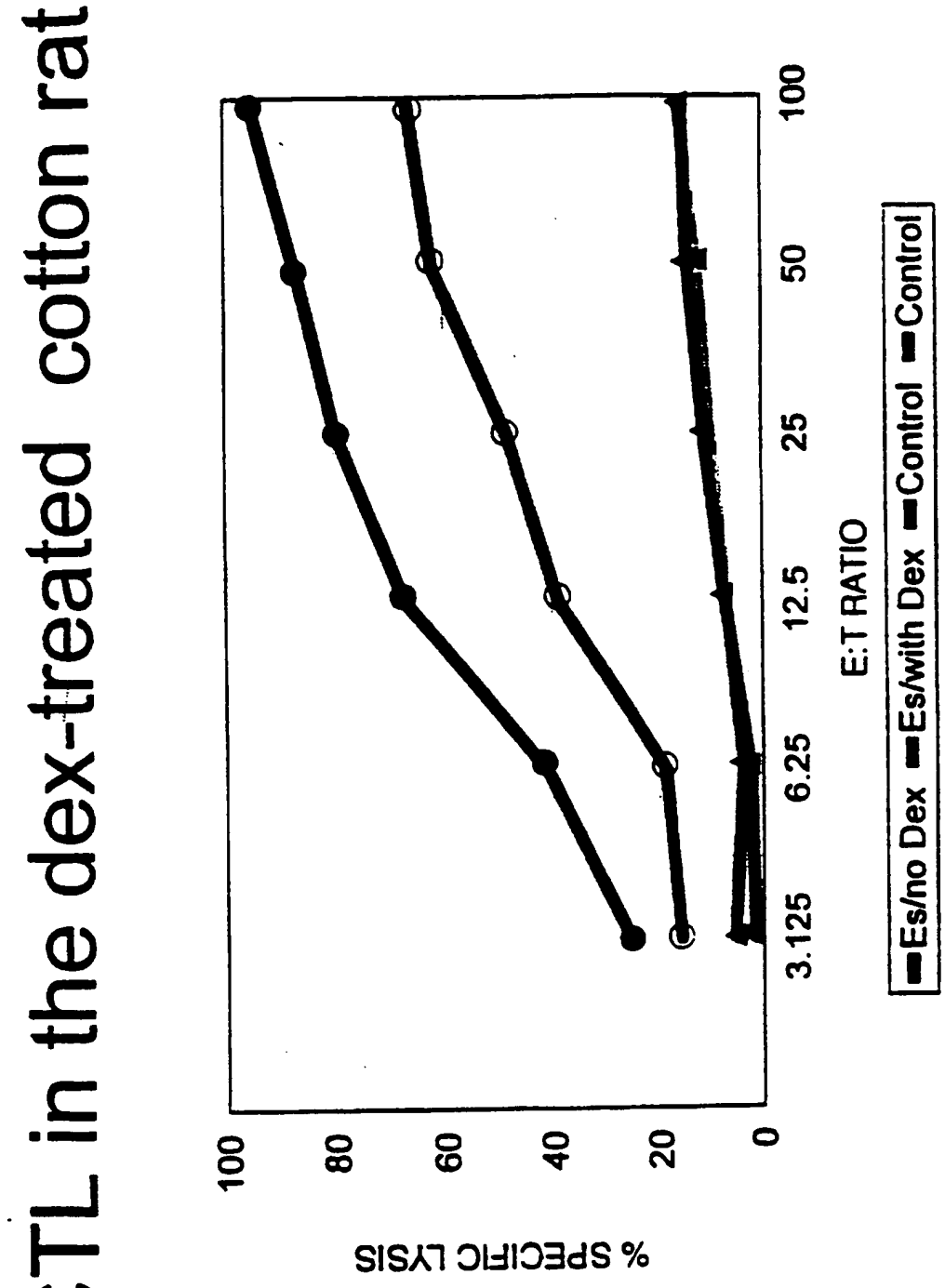

CTL in the dex-treated cotton rat
% SPECIFIC LYSIS
0
20
40
60
80
100
3.125
6.25
12.5
25
50
100
E:T RATIO
Es/no Dex
Es/with Dex
Control
Control

Fig. 13

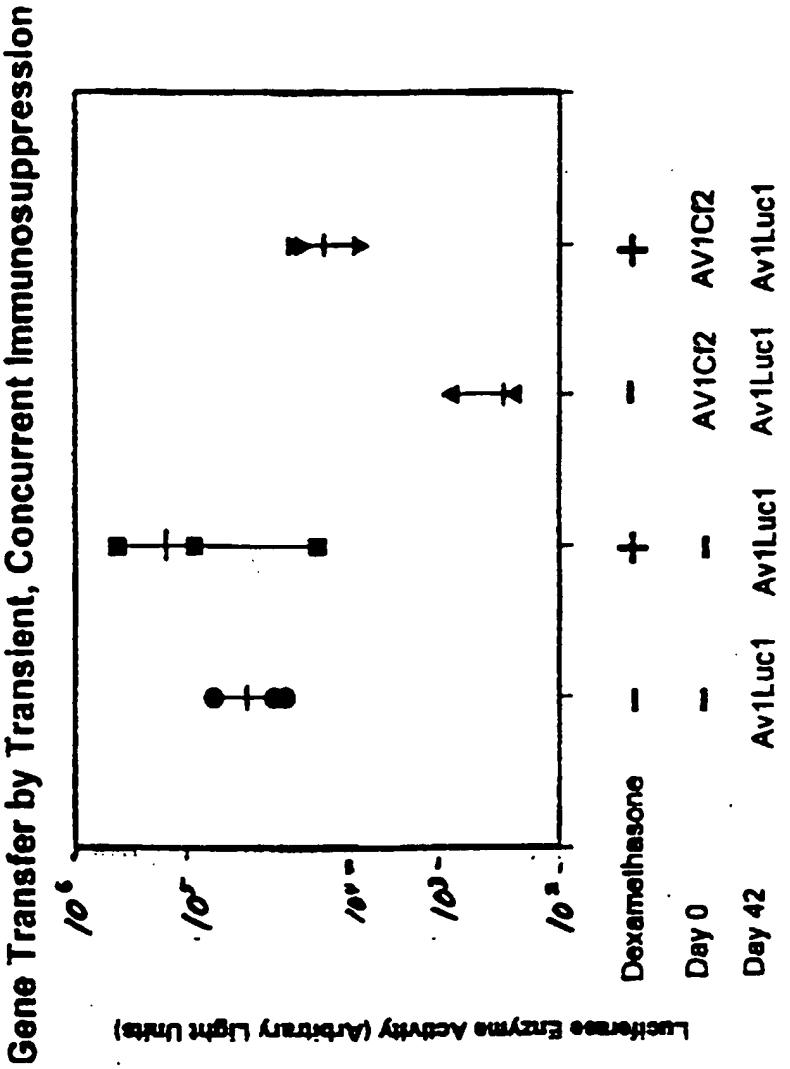

Enhancement of Repeat Av1 Vector-mediated
Gene Transfer by Transient, Concurrent Immunosuppression
Luciferase Enzyme Activity (Arbitrary Light Units)
10^6
10^5
10^4
10^3
10^2
Dexamethasone - + - +
Day 0 - - AV1Cf2 AV1Cf2
Day 42 Av1Luc1 Av1Luc1 Av1Luc1 Av1Luc1

Fig. 14

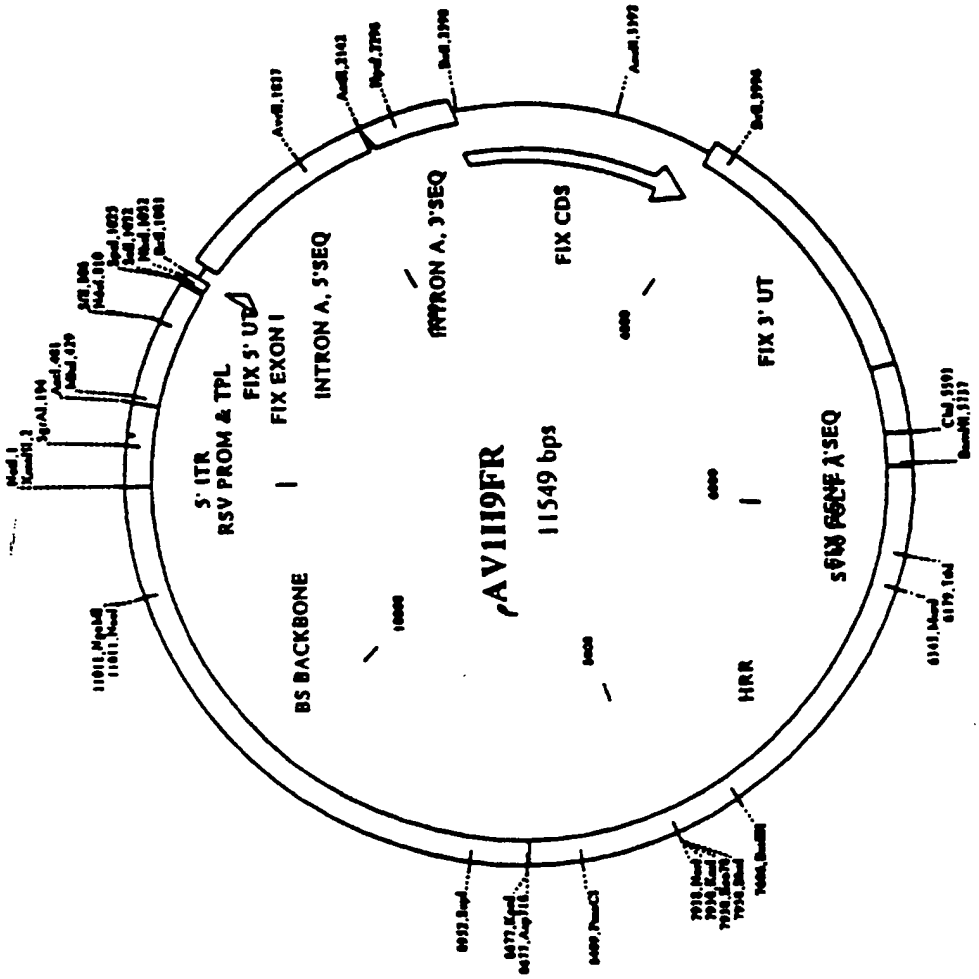

pAV1119FR
11549 bps
5' ITR
RSV PROM & TPL
FIX 5' UT
FIX EXON I
INTRON A, 5'SEQ
INTRON A, 3'SEQ
FIX CDS
FIX 3' UT
SV40 POLY A'SEQ
HRR
BS BACKBONE

Fig 15

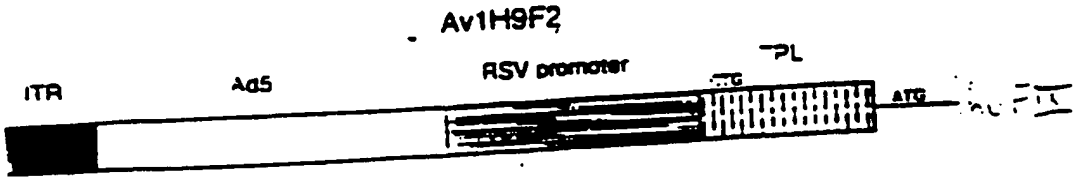
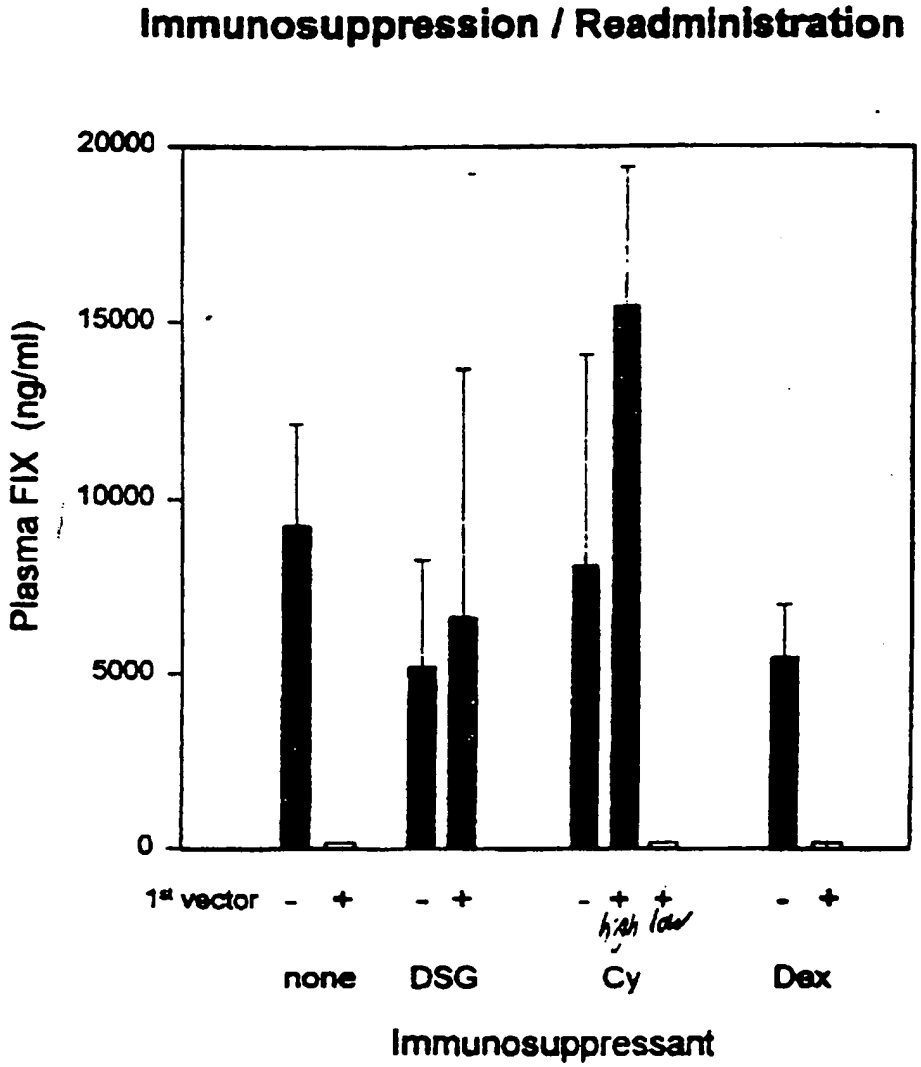


Fig. 16

Figure 17


Immunosuppression / Readministration
20000
15000
10000
5000
0
Plasma FIX (ng/ml)
1st vector - + - + - + + - +
high low
none
DSG
Cy
Dex
Immunosuppressant

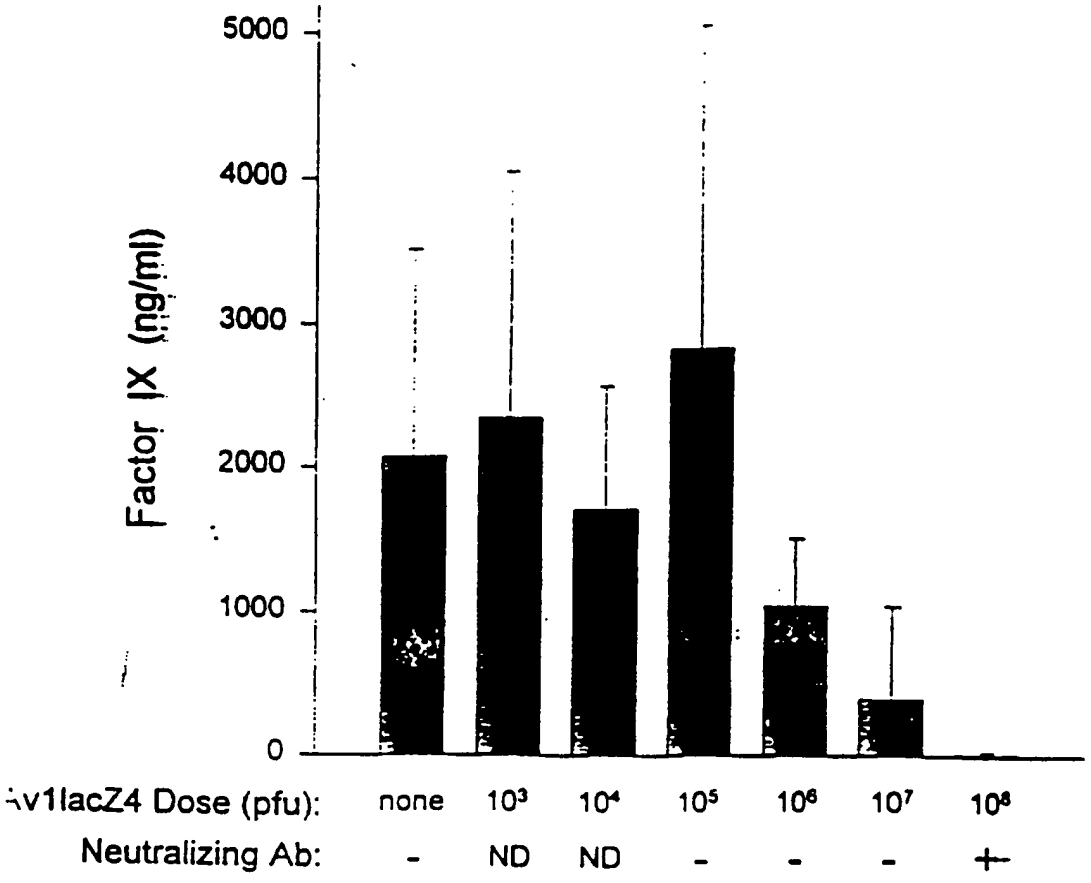

5000
4000
3000
2000
1000
0
Factor IX (ng/ml)
Av1lacZ4 Dose (pfu):
none
10³
10⁴
10⁵
10⁶
10⁷
10⁸
Neutralizing Ab:
-
ND
ND
-
-
-
+

Fig. 18

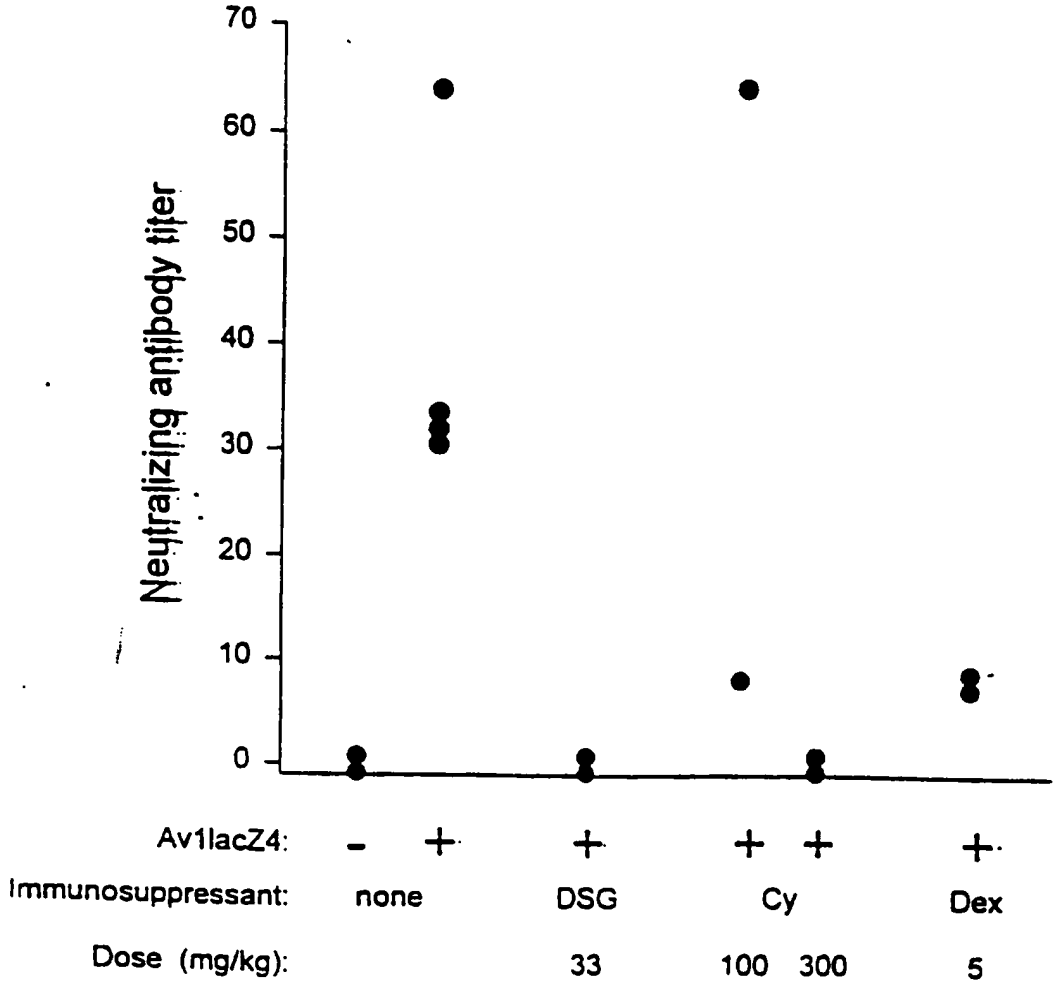

Neutralizing antibody titer
70
60
50
40
30
20
10
0
Av1lacZ4: - + + + + +
Immunosuppressant: none DSG Cy Dex
Dose (mg/kg): 33 100 300 5

Fig. 19

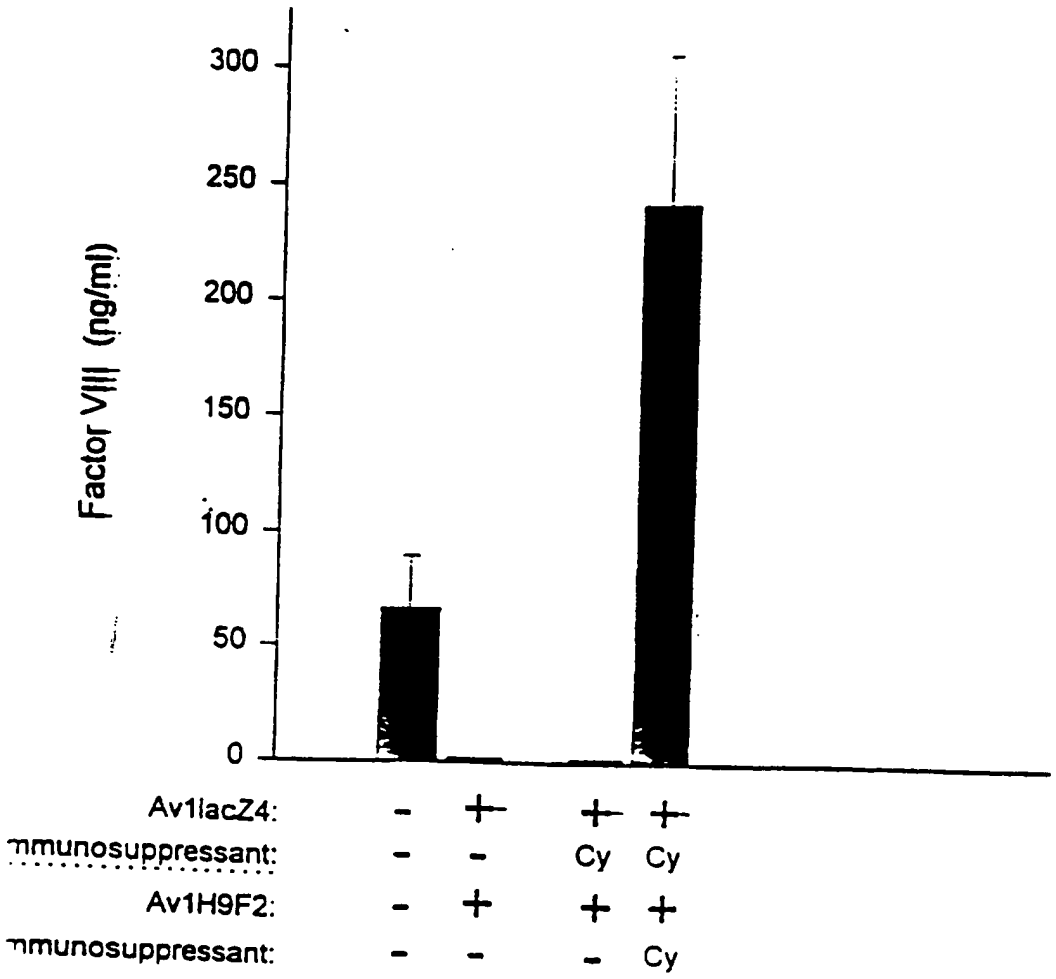

Factor VIII (ng/ml)
300
250
200
150
100
50
0
Av1lacZ4: - + + +
nmunosuppressant: - - Cy Cy
Av1H9F2: - + + +
nmunosuppressant: - - - Cy

Fig. 2C

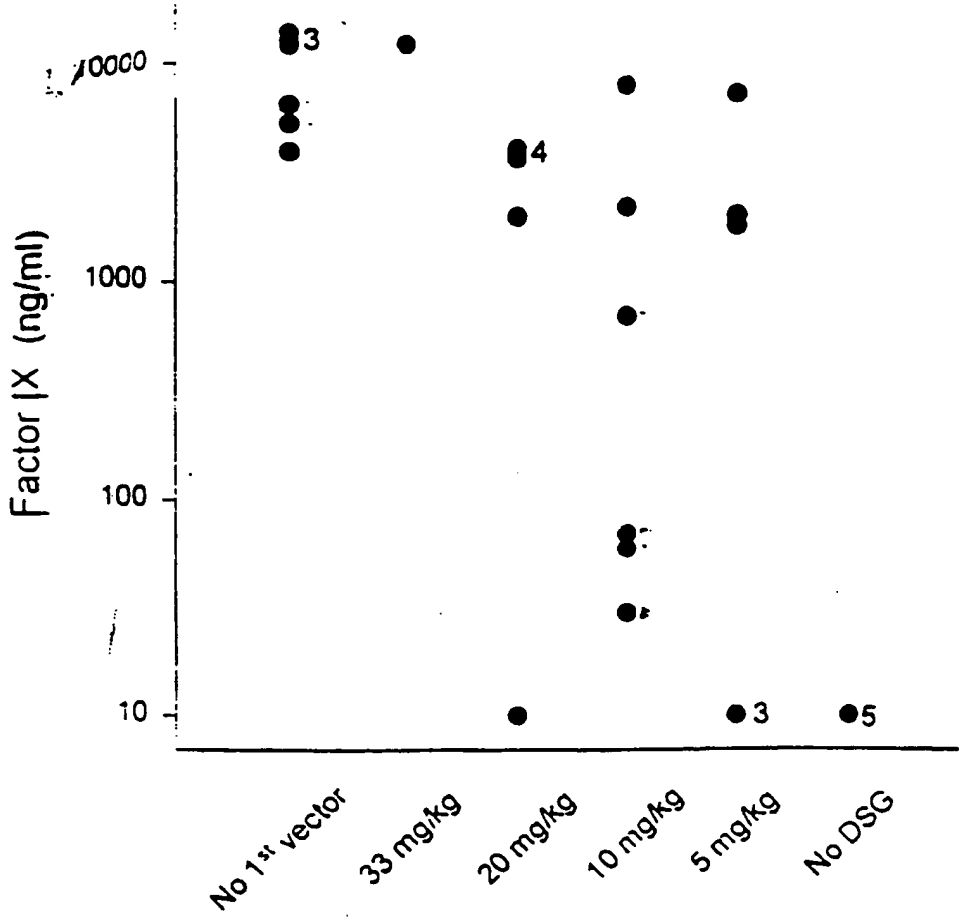

Factor IX (ng/ml)
10000
1000
100
10
3
4
3
5
No 1st vector
33 mg/kg
20 mg/kg
10 mg/kg
5 mg/kg
No DSG

Fig. 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13253

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/04, 63/00; A61K 31/70, 48/00; C12N 15/00
US CL :424/93.21; 435/172.3, 320.1; 514/44; 552/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21; 435/172.3, 320.1; 514/44; 552/574

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,290,540 (PRINCE ET AL) 01 March 1994, see entire document.	1-21
X, P ------- Y, P	Journal of Cellular Biochemistry, Supplement 21A, issued April 1995, Trapnell et al., "Pharmacologic immunomodulation enhances repeated in vivo adenovirus-mediated gene transfer", page 415, see entire document.	X 1-3 If ---------- Y 4-21
Y	Nature Genetics, Volume 6, issued January 1994, Zabner et al., "Safety and efficacy of repetitive adenovirus-mediated transfer of CFTR cDNA to airway epithelia of primates and cotton rats", pages 75-83, see entire document.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 DECEMBER 1995	08 FEB 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MICHAEL J. NEWELL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13253

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	Proceedings of the National Academy of Sciences, Volume 92, issued February 1995, Dai et al., "Cellular and humoral immune responses to adenoviral vectors containing factor IX gene : Tolerization of factor IX and vector antigens allows for long-term expression", pages 1401-1405, see entire document.	1, 4
X, P	Annals of Surgery, Volume 222, Number 3, issued September 1995, Elshami et al., "The Role of Immunosuppression in the Efficacy of Cancer Gene Therapy Using Adenovirus Transfer of the Herpes Simplex Thymidine Kinase Gene", pages 298-310, see entire document.	1, 4
X, P	Human Gene Therapy, Volume 6, issued August 1995, Fang et al., "Gene Therapy for Hemophilia B: Host Immunosuppression Prolongs the Therapeutic Effect of Adenovirus-Mediated Factor IX Expression", pages 1039-1044, see entire document.	1, 4
A, P	Science, Volume 269, issued 25 August 1995, Marshall, "Gene Therapy's Growing Pains" , pages 1050 -1055, see entire document.	1-21
Y	Nature Genetics, Volume 4, issued May 1993, Engelhardt et al., "Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses", pages 27-34, see entire document.	1-21
Y	Proceedings of the National Academy of Sciences, Volume 91, issued May 1994, Yang et al., "Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy", pages 4407-4411, see entire document.	1-21
Y	Proceedings of the National Academy of Sciences, Volume 91, Engelhardt et al., "Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver", pages 6196-6200, see entire document.	1-21
Y	Nature Genetics, Volume 7, issued July 1994, Yang et al., "Inactivation of E2a in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis", pages 362-369, see entire document.	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13253

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Medline, BIOSIS, CAPLUS
search terms: Trapnell, Yei, adenovirus, adenoviral, vector(s), immunosuppression, dexamethasone, cyclosporin, gene or genetic, transfer or therapy